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STUDIES ON THE ROLE OF ANTI-CITRULLINATED PROTEIN ANTIBODIES IN RHEUMATOID ARTHRITIS

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Studies on the role of anti-citrullinated protein antibodies in rheumatoid arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Anti-citrullinated protein antibodies (ACPA) are highly specific for rheumatoid arthritis (RA) and present in about two thirds of all patients at diagnosis. They can be detected already before disease onset and have direct pathogenic effect mediated partly through the Fc (fragment crystalizable) portion with attached Fc-glycan structure. On these grounds we aimed to further characterize ACPA's role in the pathogenesis of RA both as a risk factor and a disease biomarker. To this end we investigated ACPA occurrence in a population-derived twin cohort and ACPA in a cohort of early-untreated RA patients in relation to disease outcomes.

First we screened a large population-derived twin cohort (N= 12,590; median age 64, range 48-93 years) for occurrence of ACPA using an ACPA test used in clinical routine: the Anti-CCP2 (IgG) test. Through linking the twin cohort with the Swedish National Patient Register we identified ACPA-positive individuals without RA (N=226) and ACPA-positive patients with RA (N=124). ACPA-positive individuals without RA had lower ACPA concentration and fewer different ACPA reactivities as compared to patients with ACPA-positive RA. Heritability estimates for having ACPA with or without RA were generally lower than expected (10% (95% CI: 0-43) for ACPA without RA; 23% (95% CI: 0-45) for ACPA; and 41% (95% CI: 0-74) for ACPA-positive RA). Heavy smoking and HLA-SE associated with ACPA occurrence with and without RA. Environmental factors (including smoking) appeared to be more important than genetic in determining which individuals develop ACPA, while genetic factors (and in particular HLA-SE) had a relatively larger impact in determining which ACPA-positive individuals that will ultimately develop arthritis. We also confirmed that presence of ACPA and especially high titers of ACPA have a high diagnostic accuracy for RA in a population based setting.

Following this, we then screened a cohort of early-untreated RA patients (N=183) for occurrence of ACPA using either the Anti-CCP2 test or ELISA for detection of reactivities against specific citrullinated peptides. We demonstrated that ACPA (and especially anti-citrullinated-vimentin antibodies) associated with markers of bone loss (as measured by ELISA detection of serum RANKL and/or presence of bone erosions on radiographs of hands and feet). Treatment with methotrexate (MTX) significantly lowered both ACPA and RANKL serum levels. In a subgroup of these patients (N=59) we investigated the Fc-glycosylation patterns of serum IgG in relation to disease outcome further. A general low abundance of galactosylated glycans, partially restored by MTX treatment, was observed in the serum of early-untreated RA samples. This was more evident among future non-responders as compared to responders to MTX treatment. The galactosylation status of the IgG-Fc had good predictive value for MTX response.

In conclusion we showed that environmental as well as genetic factors are important for ACPA occurrence, which in turn has a high diagnostic accuracy for RA. In early-untreated RA, ACPA associate with bone loss and is modulated by methotrexate treatment. Further, Fc-glycosylation patterns of antibodies are generally altered in early-untreated RA and might serve as a predictive factor for therapeutic response.

LIST OF SCIENTIFIC PAPERS

- I. **Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins**
A. Haj Hensvold, P. K. E. Magnusson, V. Joshua, M. Hansson, L. Israelsson, R. Ferreira, P-J. Jakobsson, R. Holmdahl, L. Hammarström, V. Malmström, J. Askling, L. Klareskog, A. I. Catrina
Annals of Rheumatic Diseases, 2015;74:375-380
- II. **How well do ACPA discriminate and predict RA in the general population – a study based on 12,590 population-representative Swedish twins**
A. Haj Hensvold, T. Frisell, P. K. E. Magnusson, R. Holmdahl, J. Askling, A. I. Catrina
Recommended for publication, Annals of Rheumatic Diseases
- III. **Serum RANKL levels associate with anti-citrullinated protein antibodies in early untreated rheumatoid arthritis and are modulated following methotrexate**
A. Haj Hensvold, V. Joshua, W. Li, M. Larkin, F. Qureshi, L. Israelsson, L. Padyukov, K. Lundberg, N. Defranoux, S. Saevarsdottir, A. I. Catrina
Arthritis Res. Ther. 2015;17:239
- IV. **IgG Fc galactosylation changes and predicts response to methotrexate in early rheumatoid arthritis**
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*Manuscript. *Equal contribution*

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LIST OF ABBREVIATIONS

ACPA	Anti-Citrullinated Protein Antibodies
ACR	American College of Rheumatology
Anti-CCP	Anti-Cyclic-Citrullinated Peptide antibodies
Cit	Citrullinated
CI	Confidence interval
CRP	C-Reactive Protein
DAS28	Disease Activity Score - 28 joints
sDMARD	Synthetic Disease-Modifying Anti-Rheumatic Drug
DZ	Dizygotic twin
ESR	Erythrocyte Sedimentation Rate
EULAR	European League Against Rheumatism
Fab	Fragment antigen binding
Fc	Fragment Crystallizable
FDR	First Degree Relatives
HAQ	Health Assessment Questionnaire score
HLA	Human Leukocyte Antigen
Ig	Immunoglobulin
IL	Interleukin
MTX	Methotrexate
MZ	Monozygotic twin
NPV	Negative Predictive Value
OPG	Osteoprotegerin
OR	Odds ratio
PPV	Positive Predictive Values
RA	Rheumatoid Arthritis
RANKL	Receptor-Activator of Nuclear factor Kappa-b Ligand
RF	Rheumatoid Factor
SE	Shared epitope
TNF	Tumor Necrosis Factor

1 INTRODUCTION

1.1 RA - AN INFLAMMATORY CHRONIC DISEASE

Rheumatoid arthritis (RA) is an inflammatory chronic joint disease typically affecting small joints in hands and feet with risk for functional disabilities and further comorbidities. Population prevalence in Sweden is estimated to be 0.8% (range 0.1-2.2% depending on age) and annual incidence rate is approximately 40/100 000 (range 10-90/100 000 depending on age) [1, 2]. The understanding of RA has evolved with the development of modern medicine, increasing research interest and an increase in treatment possibilities. One important step was the formulation of the first modern classification criteria for rheumatoid arthritis 1957 [3]. The aim of these criteria, that included criteria about pain and swelling of joints and rheumatoid factor (RF), was to facilitate research, in a clinical setting, by homogenize the RA patient group and create demarcations to other rheumatologic diseases. Since then, two to large updates of the RA criteria have been implemented. The revision year 1987 made more precise definitions of the criteria with a focus of hand and small joint involvement as characteristic for RA [4]. In 2010, the most recent revision was implemented and aimed to create criteria focusing on features at earlier stages of disease applicable to patients with arthritis of shorter duration [5]. Presence of autoantibodies, not only RF but also anti-citrullinated protein antibodies (ACPA), was included as a new criterion. Presence of bone changes were no longer included, but a new definition of erosions typical of RA were later proposed (cortical bone breaks in at least three separate joints in hands and feet) to be applied in patients not fulfilling 2010 RA criteria [6].

1.2 PATHOGENIC TRAITS IN RA

Once established, RA is a disease mainly affecting the joints with chronic synovial inflammation and associated bone destruction.

The main pathogenic traits of RA are joint inflammation and bone destruction. Joint inflammation is characterized by synovial hyperplasia with local accumulation of inflammatory cells (macrophages, dendritic cells and lymphocytes) and increased production of cytokines. The numerical dominating leukocyte group is the cell of the myelomonocyte lineage and especially activated macrophage locally producing important pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-6 [7]. Synovial fibroblasts are expanded in the synovial lining and the sublining and can invade the cartilage and bone and secrete pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating

factor (GM-CSF) and IL-6 [8]. T-cells, B-cells and plasma cells are present in the inflamed synovia and can occasionally form follicular-like structures [9].

Beside joint inflammation, cartilage and bone destruction are other important pathogenic trait of established RA. Approximately half of the patients with symptom duration of less than one year have radiographic bone and cartilage damage in small joints at diagnosis [10, 11]. Bone homeostasis is maintained by a balance between bone formation and degradation governed by the receptor-activator of nuclear factor kappa-b (RANK) -RANK ligand (RANKL) - osteoprotegerin (OPG) system [12, 13]. The binding of RANKL to cellular RANK receptor stimulates osteoclastogenesis and promotes maturation of osteoclasts and bone resorption. RANKL is expressed in three different forms; a membrane form, a secreted and a secreted cleaved form. Secreted RANKL is present in peripheral blood both in a free form in low concentrations and a bound form (in complex with OPG or other serum binding proteins) in higher concentrations [14]. Soluble RANKL is a trimer that binds RANK and via receptor clustering leading to intra-cellular signaling and modified gene expression. RANKL-RANKL signaling in precursors will drive osteoclast differentiation and also activate and promote survival of osteoclast [13]. The effect of RANKL is counterbalanced by OPG that functions as a decoy receptor to RANKL. RANKL is expressed in the synovial tissue and serum in RA patients [11, 15-19] and a correlation between RANKL and bone destruction in RA has been suggested [15, 20-22]. Additionally, treatment with Denosumab - an anti-RANKL drug - inhibits bone destructions (erosion and bone density) independent of effects on inflammation [23, 24].

1.3 AUTOANTIBODIES ASSOCIATED WITH RA

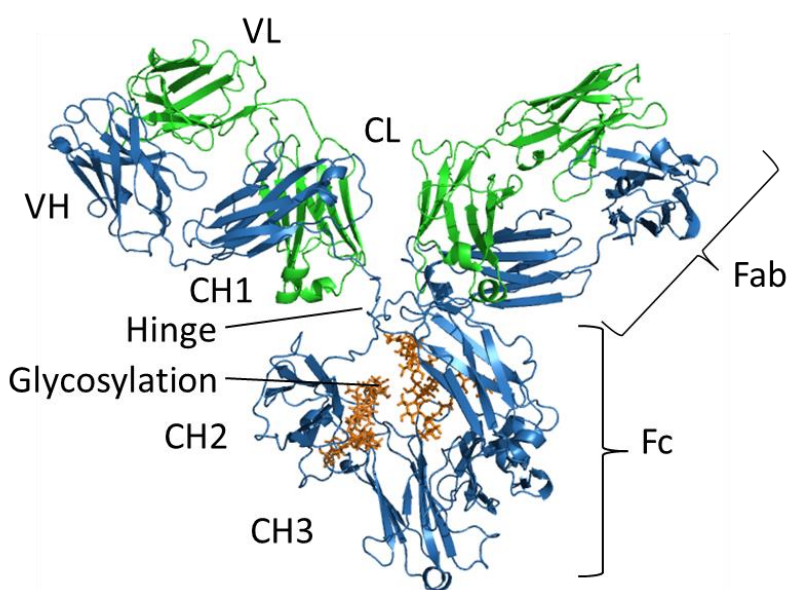
A third pathogenic trait in RA is the presence of autoantibodies (antibodies reacting against own tissue targets). Autoantibodies are markers of disease, present in a majority of all patients, with possible roles in the pathogenesis of autoantibody-positive RA.

1.3.1 Antibodies

Antibodies are secreted by B-cells, into lymphatic tissue and spread to surrounding tissue, lymph vessels, blood stream and mucosal sites. Together with albumin, antibodies make up the largest part of the protein content in serum [25]. Antibodies are also expressed on surface cell membranes on B-cells creating a B-cell receptor as well as bound to receptors on other immune cells. Antibodies are produced as part of an induced immune response to e.g. infections and have the capability to bind specifically to macromolecules from infectious

microbes and thus preventing them from spreading. The antibody is made of two light chains and two heavy chains and make up a quite large molecule (IgG: 150 kilodalton) with two variable regions and several constant regions (figure 1) [26]. Structurally, the antibody is composed of an antigen binding fragment (Fab) and a crystallisable fragment (Fc). The Fab contains the variable regions and creates a large repertoire of antibodies recognizing different antigens. The Fc portion makes up the structural framework and variation in the Fc creates different classes of antibodies: IgA, IgD, IgE, IgG, IgM.

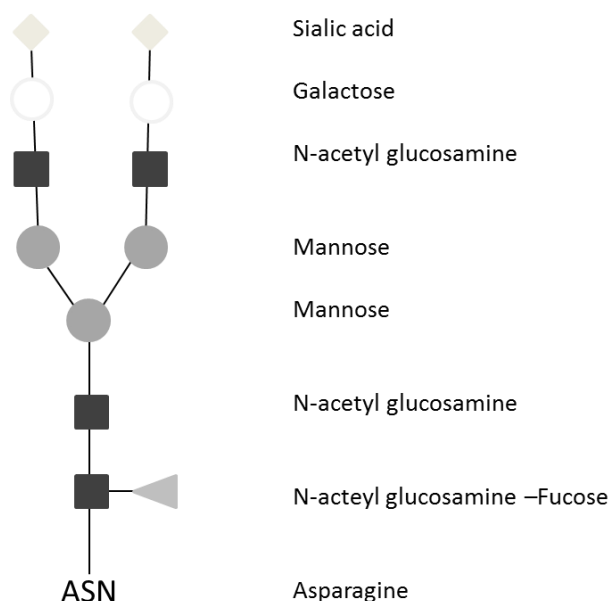
Figure 1: Overview of IgG antibody structure



Light chain in green, heavy chain in blue and N-linked glycan in orange. Abbreviations: variable light (VL); variable heavy (VH); constant light (CL); constant heavy 1-3 regions (CH1-3); antigen binding fragment (Fab); crystallisable fragment (Fc). Figure borrowed with permission [27]

Antibodies have several glycosylation sites and the number of sites and location vary between classes and subclasses [28, 29]. IgG_{1/2/4} have two glycosylation sites on the constant regions of the heavy chain (so called Fc-glycans) where two N-linked glycans (figure 2) are attached. IgG₃ has one additional glycosylation site for an N-linked glycan and IgA₁ also have sites for hydroxyl-group-linked glycans [28, 29]. The N-linked glycan attach to the nitrogen in asparagine amino acid and constitute a biantennary heptasaccharide with N-acetyl glucosamine and mannose residues. In addition, varying numbers of fucose, galactose and sialic acid can be attached.

Figure 2: Fully sialylated and galactosylated N-linked glycan



The N-linked Fc-glycan is bound to a hydrophobic area and protrude towards the interstitial space between the pairs of the constant regions, where it interact with the opposite N-linked glycan on the other chain and creates an ‘open’ structure for Fc-receptor (FcR) binding [26, 30]. Depending on variable expressions of the amino acids in the Fab region, N-glycans can also be positioned on this part of the molecule.

1.3.2 B-cells and autoreactivity

One of the major tasks for the immune system is to find a balance between recognition of pathogens and avoidance of reactivity to self-molecules (autoreactivity and autoantibodies). In the bone marrow, where the B-cell maturation starts, B-cells are matured in an environment exposing the immature B-cells for self-tissue antigens. One of the first steps for avoidance of autoreactivity secures only one antibody specificity per B-cell. Only properly functioning B-cells with a possibility to signaling through their B-cell receptor (BCR) will be selected to proliferate. In contrast; B-cells that react too strong to local antigens will be induced to further receptor editing of light chain, anergy or apoptosis [31]. B-cell clones reacting weakly with self-antigen are ignored and migrate to the periphery. In patients with RA, the mechanisms of early filtering out autoreactive B-cells is suggested to be impaired [31]. Even in healthy individuals the repertoire of circulating autoreactive B-cells ready for activation and differentiation is not negligible [32].

The mechanisms in the bone marrow are supported by further peripheral mechanisms for avoidance of autoreactivity. To proliferate and differentiate autoreactive B-cells clones need

stimulation and selection. Response from B-cells with maturation and differentiation of B-cells and increased antibody production, can be independent or dependent of help from T-cells, depending on antigen and co-occurring stimuli. Blood-borne pathogens, engaging multiple BCR, can create sufficient stimuli for B-cell activation without help from T-cells [33]. DNA or RNA from pathogens can activate B-cells via stimulation of BCR and toll-like receptors and induce antibody secretion [34]. However, T-cell- dependent activation of B-cells is typically needed for efficient activation of B-cells, to induce class switch and affinity maturation through somatic hypermutation and selection, and generation of memory and long-lived plasma cells. Activated B-cells that have encountered antigen will interact with activated CD4⁺T-helper-cell and be exposed to CD40-ligand in the border of the lymphoid follicle [35]. Some B-cell clones will differentiate to short-lived plasma cells and some will migrate into the follicle and re-engage with antigen presented by follicular dendritic cells and be re-stimulated by T-follicular helper cells. Inside the follicle a germinal center, in which B-cells will terminally differentiate and become long-lived memory or plasma cells, will be formed. Some B-memory cells and long-lived plasma cells will remain in the lymph noduli and others will circulate respectively migrate to the bone marrow [36].

1.3.3 ACPA, RF and other autoantibodies in RA

A RA patient typically has normal levels of antibodies in the blood, but compared to healthy individuals, a majority of the RA patients have (often several) specific autoantibodies targeting endogenous molecules present in higher concentration (such as RF and ACPA). In ACPA-positive RA, estimations suggest that up to 1.5% of the total IgG amount in peripheral blood is constituted of ACPA and up to 25% of synovial joint fluid B-cells and recombinant expressed antibodies from synovial B-cells have ACPA reactivity [37-40]. In comparison the amount of antibodies towards tetanus and influenza, induced by vaccination and or infections, is less than 0.2% of the total IgG level [41].

1.3.3.1 RF

RF was identified already in 1940 by its ability to aggregate sheep cell erythrocytes, treated with anti-sheep erythrocyte antibodies [42, 43]. About 70% of RA patients are RF positive while 1- 5% of healthy controls are considered to have abnormal high levels of RF. RF is also present in increased proportion in patients with other disease like other rheumatic diseases such as SLE, or infections [44, 45]. RF can be of IgM, IgG, IgA or IgE isotype and is directed towards the Fc-portion of IgG. The epitope/s is not known but RF bind immunoglobulins

from different origin (sheep, mouse, rat, goat, rabbit, and other), primary IgG isotypes and preferable subclasses 1,2 and 4 [46].

1.3.3.2 ACPA

In 1964 antibodies targeting keratohyalin granules in buccal mucosa cells called antiperinuclear factor were identified in sera and synovial fluid from RA patients [47] and later shown to recognize citrullinated (cit-) peptides [48]. Cit-proteins are formed during an enzymatic process (called citrullination) catalyzed by peptidylarginine deiminase (PAD) enzymes in which an arginine residue is converted to citrulline. It occurs in different tissues and during different phases of health and disease [49, 50] and is present in the inflamed synovial tissue [51, 52]. It is believed that citrullination of proteins allows a better binding to particular human leukocyte antigen (HLA) II molecules on antigen presenting cells, leading to a more efficient presentation of cit-antigens [53].

A diagnostic ELISA (enzyme-linked immunosorbent assay) test for prevalent and early RA using a synthetic cit-filaggrin-derived peptide (Anti-CCP test) was developed for use in clinical practice in year 2000 [48, 54]. The Anti-CCP test used in sera had 68% sensitivity for prevalent RA and 98-99% specificity (among disease and healthy controls) [54]. Anti-CCP antibodies were also present in few patients with other rheumatic diseases such as SLE and infections but had higher specificity in comparison with RF [44, 45, 54]. An antibody detected by an Anti-CCP test includes a large range of antibodies with different reactivities. Targets for these antibodies (such as apolipoprotein, biglycan, clusterin, collagen type II, alpha-enolase type 1, fibrinogen, histone, tenascin-C, vinculin and vimentin) have been identified and there are often several cit-targets for each protein [55-62].

ACPA is presented as IgG, IgM, and IgA isotypes. Among IgG ACPA (typically measured isotype), IgG₁ is the most common subclass, but IgG₄ seem to be relatively increased [63-66].

ACPA show a certain degree of cross-reactivity and have been suggested to be polyreactive and able to bind different cit-peptides with similar or different affinity [37, 67]. However, both cross-reactive anti-cit antibodies as well as no cross-reactive anti-cit antibodies can be found in RA patients. Blocking experiments with cit-peptides (cit-alpha enolase type 1, cit-vimentin, cit-fibrinogen and cit-collagen peptides) have shown a variable decrease (from a few percent to 100%) in sera reactivity [55, 68, 69]. ACPA do not, however, crossreact with native unmodified (not cit) proteins [37]. Antibodies against native proteins might coexist with those against cit-proteins and in some cases even antedate the development of ACPA (as

for some vimentin and filaggrin peptides) [70]. Typically in RA, serum reactivity toward the cit-peptides is higher than that toward the unmodified peptide [57].

Regarding specific information about specific B-cells and autoantibodies in RA patients, a study of B-cells derived from peripheral blood in RA patients do find a higher proportion of autoantibody (ACPA) positive class-switched memory- and plasma-cells compared to naïve [71]. Similarly, in the synovial tissue the majorities of present B-cells express IgG and are of a memory type. Plasma cells expressing IgG in the synovia are also present, which could be a result of local antigen-specific activation. Further there are signs of clonal expansion of B-cells and migration within the synovial tissue [72]. Studies of monoclonal ACPA suggest, that differentiation of ACPA-specific plasma cells might occur in germinal center structures due to the presence of non-random somatic hypermutations [37, 73].

1.3.3.3 Other autoantibodies

Apart from ACPA, antibodies against posttranslational modified proteins (such as anti-carbamylated protein antibodies [74-76], anti-malondialdehyde-acetaldehyde [77], anti-acetylated vimentin antibodies [78]) as well as antibodies directed against native proteins (such as those targeting the nuclear ribonucleoprotein A2 [79] and anti-BiP [80-82], calpastatin [83, 84], cartilage antigens [55], hinge region in antibodies [85], and PAD [86, 87] have been described in RA patients.

The large number of antibody reactivities as well as occurrence of different isotypes creates a large heterogeneity of antibody patterns in individual RA patients.

1.3.4 Effector functions of ACPA

Overall antibody effector functions are defined by the structure of Fc, and the antigen-specificity by the Fab. Soluble antibodies bind soluble antigens via Fab and form immune complexes (IC). The Fc region can induce cellular response: antibody dependent cellular cytotoxicity and antibody mediated phagocytosis induce activation of complement cascade and neutralization of pathogens. In this section we will short describe effector functions of ACPA.

In some mice models ACPAs are able to induce mild arthritis and enhance arthritis and in others not [88-91]. Cellular studies have suggested binding of ACPAs and ACPA containing immune complexes to Fc receptors as well as specific binding of ACPA through Fab portions, resulting in several effector functions of ACPA such as cytokine release (TNF and

IL-8) [90, 92-96], osteoclast differentiation, fibroblast migration [93, 97, 98], platelet activation [99], mast cell activation, neutrophil extracellular traps and complement activation [96, 100-102].

Among the best-studied effector functions of ACPA both in vitro and in animal models is the capacity of these antibodies to promote osteoclast activation and bone destruction. These findings in cell cultures and mice are supported by observations indicating that ACPA associates with bone destruction in ACPA positive individuals with or without arthritis [103-106].

1.3.5 Antibody glycosylation and RA

Changes in the distribution pattern of IgG glycans are present in RA patients with an increase in the agalactosylated types (lacking galactose) in both serum and synovial fluid [63, 107-111]. It has been suggested that general changes in the activity of the galactosyltransferase might be responsible for this distribution shift [112].

Agalactosylated IgG intrinsically lack both galactose and sialic acid and expose mannose residues, potentially promoting complement activation through mannose-binding-lectin-pathway [113, 114] or alternative and classical pathways [115]. Recently, it has been suggested that heat-aggregated IgG IC lacking sialic acid (bound to galactose residue) induce osteoclastogenesis better compared to IC with sialic acid or degalactosylated IC or monomeric IgG lacking sialic acid [116]. In parallel, human data in the same study suggest, that in RA patients and ACPA-positive healthy individuals' low proportions of glycan residues containing sialic acid and galactose on IgG₁, are associated with lower bone mass in the hand [116]. Agalactosylated IgG is also previously known to moderately correlate with disease activity and acute phase reactants [109, 117, 118]. Interestingly, the glycan pattern for ACPA seems to be changed compared to other IgG [63, 107, 109, 119].

1.4 STUDIES ON THE DEVELOPMENT OF ACPA IN RA

Already in the 60ies studies reported that RF preceded the onset of RA (reviewed by del Puente et al 1998) [120]. Throughout the following decades reports kept showing increased incidence of RA among RF positive tested prior to onset, but the number of incident cases were most often very small [120-124]. The first report indicating that ACPA (typically in RF-positive samples) also preceded RA onset came 1992 [125]. Some years later, the occurrence of ACPA, measured by Anti-CCP (IgG) test using the cit-peptide described by Schellekens year 2000 [54], was investigated in a nested-case control study among blood donors (N=79

cases and 2,138 controls) with serial blood samples (in median 13), antedating the disease onset, during a long follow-up (in median 7.5 years; range 0.1-15) [126]. This investigation showed, that preceding symptom onset IgM-RF, IgG-ACPA, IgM-RF and/or IgG-ACPA were present in about one third, two fifth and half of all the cases respectively. Analyzing sensitivity stratified by number of years prior to symptom onset showed that the sensitivity of the Anti-CCP test for RA was increased compared with the sensitivity of IgM-RF. At a similar time point, occurrence of ACPA measured by a manufactural produced Anti-CCP2 (IgG) test using an 'updated' cit-peptide (CCP2) was investigated in another nested-case control study among population-derived research subjects (N=83 cases and 382 controls) [127]. They also found high sensitivity for Anti-CCP2 when analyzing in average one sample per case from a short follow-up period to onset of symptom (median 2.5 years (range 0.1-21) years). Similar to Anti-CCP2, IgA-RF was a common preceding antibody. A more recent updated nested-case study among the same population-derived research subjects (N=386 cases and 1,305 controls) with serial blood samples available (in median 2) during a longer follow-up (in median 7.4 years (range 0.1-30) years) found that preceding symptom onset, IgG-ACPA were present in about one third of all the cases, in correspondence with the first report [58]. ACPA as a preceding autoantibody were later confirmed in at least six other Pre-RA cohorts with samples collected prior to RA diagnosis and/or symptom onset and with an overall total about 1100 included Pre-RA patients (table 1)[62, 128-133]. IgG-ACPA precede RA onset in median in 31% (range 22-61%) of investigated Pre-RA patients (note this prevalence do also include ACPA-negative patients at diagnosis). The reported difference in occurrence between the Pre-RA cohorts is probably influenced by the difference in patient composition (the cohort studied by Majka et al is almost exclusive seropositive at onset), the number of samples studied per patient and difference in test methods. Overall, IgG-ACPA seems to frequently precede diagnosis among those that at onset are ACPA-positive but many Pre-RA cohorts lack samples from time-point of diagnosis [58, 128].

Table 1: Pre-RA cohorts and prevalence of ACPA measured by Anti-CCP (IgG) test

Author (year) Origin	Nielen (2004) J Breemen Institute and Sanquin blood bank, Holland.	Rantapää-Dahlqvist (2003) Medical Biobank of Northern Sweden and Umeå University Hospital.	Brink (2013) Medical Biobank of Northern Sweden and Umeå University Hospital.	Majka (2008) Serum Repository and Walter Reed Army Medical Center, USA.	Jørgensen (2008) JANUS Serum Bank and Oslo RA Registry, Norway.	Chibnik (2009) Nurses' Health Study I+II, Screening Questionnaire, USA.	Arkema (2013) Nurses' Health Study I+II, Screening Questionnaire, USA.	Turesson (2011) Malmö Diet Cancer Study and Swedish patient register.	Fischer (2015) Study into Cancer and nutrition and health registers, Italy and Spain.
N cases (controls)	79 (2,138)	83 (382)	386 (1,305)	83 (83)	49 (245)	93 (279)	192 (567)	169 (169)	103 (309)
N samples cases (controls)	1,188 (2,358)	98 (382)	717 (1,305)	243 (83)	49 (245)	93 (279)	192 (567)	169 (169)	103 (309)
N year in median (range/IQR¹), from sampling to symptom or RA onset	7.5 (0.1–15)	2.5 (1–5 ¹)	7.4 (3–13 ¹)	6.6 (0.1–14)	9.3 (0.3–23)	5.6 (0.3–12)	7 (0.3–17)	5 (1–13)	7 (2–16)
Case, female	62%	83%	82%	41%	63%	100%	100%	79%	78%
Cases, age median (range/±SD²/IQR¹)	51 (11 ²)	54 (27–68)	57 (49–64 ¹)	40 (20–66)	50 (24–83)	60 (8 ²)	60 (10 ²)	63	51
Cases RF+%; CCP+%	61% ; NR	73% ; 70%	NR ; 75%	81% ; 68%	NR	53% ; NR	NR	70% ; NR	56% ; NR
Anti-CCP test (manufacture)	CCP (in house)	CCP2 (Eurod)	CCP2 (Eurod)	CCP2 (Diastat)	CCP2 (Eurod)	CCP2 (Diastat)	CCP2 (Biorad)	CCP2(in house)	CCP2 (Diastat)
% any time Anti-CCP+ in pre-RA samples	41	34	34	61	31	28	12	22	23
% any time RF+ (isotype) in pre-RA samples	28 (IgM)	34 (IgA) 19 (IgM)		57 (all)	20 (IgA) 21 (IgM)			19 (IgM)	22 (IgM)

Abbreviations: number (N); positive (+); not reported (NR); Eurodiagnostica (Eurod) interquartile range 3–4th (IQR¹); standard deviation (SD²).

Studies in Pre-RA cohorts have shown that in the preceding phase IgA and IgM ACPA are also common. The development of IgG ACPA, which is most common, seems to be first, then IgA, and then IgM [134, 135].

The character of the antibody response and stability of the response can reveal information about how mature the response is once detected positive in the peripheral blood. However many Pre-RA cohorts have only a single sample per Pre-RA patient but there are two Pre-Ra cohorts with serial samples (about 2-13) per Pre-RA patient, where they report persisting IgG Anti-CCP positivity in 68-81% [58, 126].

At median five years prior to onset, the concentration is in average four times the cut-off and in the range of two to nine times the cut off [131]. A marked increase in concentration of Anti-CCP starting 1.5/2-3/5 or 6-8 years prior onset have been found, when analyzing concentrations in samples stratified by time to diagnosis [56, 62, 68, 126, 127]. The increase in titer is also paralleled by a gradually increasing frequency of Anti-CCP positive individuals closer to onset. Once a diagnosis of RA is made the titers of ACPAs are quite stable with subtle changes and a low frequency of seroconversion following treatment of early RA [136-139].

The increased in Anti-CCP titer closer to diagnosis is also paralleled by an increase in the number of detected ACPA reactivities [56, 58, 62, 68, 133]. Antibody reactivity to cit-alpha-enolase-1 and Anti-CCP2 seem to emerge later than to certain cit-vimentin and cit-fibrinogen peptides [58, 62]. These studies have also described a notable individual difference in the development and presence of ACPA reactivities. After an RA diagnosis the proportion of patients testing positive for antibodies toward cit-enolase, cit-vimentin and cit-fibrinogen declined, particular those toward cit-vimentin, with an overall lowering concentration for all tested reactivities during the first year of treatment [137].

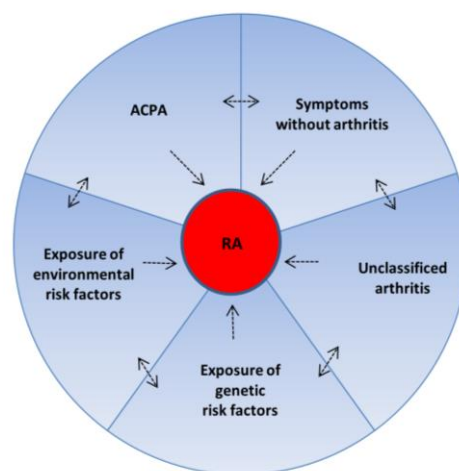
The maximum time from first occurrence of Anti-CCP and the onset of disease is reported to be in the interval of 25-13 years [58, 62, 126-128, 132]. The median time from an Anti-CCP positive sample to onset were 5-6 years, but most of the studied cases only had one Pre-RA sample [58, 126, 128, 129]. Information from Pre-RA cohorts about time lag can be compared with information from cohorts of patients with joint symptoms (lacking arthritis) and autoantibodies. In patients referred to rheumatologist with positive RF or ACPA test and joint symptoms (in median duration of 12 months (IQR 8-46)) the median observed time to arthritis onset is reported to be 12 months (IQR 6-27) when followed by a doctor in median 32 months (IQR: 13-48)[140]. Similar results were also found in two other smaller cohorts, where joint symptoms were present in median in 20 months and onset of arthritis after 8-12 months follow up [141, 142]. In summary, these results suggest a longer phase with preceding antibodies (at least five years), a shorter phase of localized joint symptoms (one year) and then rather soon (within one year) arthritis development.

Overall, the signs of inflammation measured by acute phase reaction accompanying antibody development before disease onset seem to be mild with a majority of the studies reporting no significant difference in CRP (C-Reactive Protein) or ESR (Erythrocyte Sedimentation Rate) in Pre-RA samples compared to controls [143-146]. Similarly no clear cut differences in cytokine levels have been reported [132, 144, 147]. However, many studies report a trend, of gradual increase of acute phase reactants and cytokines closer to disease onset [56, 143, 145, 147].

1.5 RISK FACTORS FOR AND PHASES OF ACPA-POSITIVE RA DISEASE DEVELOPMENT

Investigating risk factors involved in the development of ACPA-positive RA can be one link to understand underlying pathogenesis. The development of ACPA-positive RA can be described as a gradual (or stepwise) process of acquiring increased susceptibility to disease, following exposure to environmental and genetic risk factors for RA. Individuals can pass through a phase of systemic autoimmunity (such as ACPA-positivity), a phase of symptoms without clinical arthritis and a phase of unclassified arthritis leading to development of RA [148]. This is suggested terminology for defining specific subgroups during different phases of disease development in prospective studies. The order of the phases is not strict; one can skip some of the phases or pass through them at once, or go through the phases but never develop RA (figure 3).

Figure 3: Model of phases of RA disease development prior to onset



1.5.1 Risk factors for RA

Much of the knowledge about risk factors for developing RA comes from studies of incident RA or prevalent RA. I therefore start by presenting an overview of suggested risk factors for RA and ACPA-positive RA in particular, and then summarize results from studies investigating phases preceding RA onset.

1.5.1.1 Genetic factors

The most well established risk factor for ACPA-positive RA is the shared epitope (SE) alleles of human leukocyte antigen (HLA) [149-151]. HLA-SE alleles are a group of alleles expressed by the HLA-DRB1 gene at chromosome 6p21, coding for DRB1 chain molecule. The DRB1 chain is part of a heterodimer HLA-II molecule (together with the alpha chain), which is expressed on antigen-presenting cells and is central for antigen-presentation to CD4+ T-helper-cells. Several hundred different HLA-DRB1 alleles occur in a population, and some are rare (<1%) and other very common (up to 20%) [152]. The group of alleles considered HLA-SE (*01:01; *01:02; *04:01; *04:04; *04:05; *04:08; *10:01) share similarities in a certain amino-acids (position 70-74 in the third region of the DRB1 beta chain) and are involved in the peptide-binding groove. The association between ACPA-positive RA and the individual alleles varies in strength and the associations with alleles *04:01 (that is common) and *04:04 are particularly strong [153]. Studies on HLA-SE binding capacity have shown, that these alleles present cit-peptides more efficiently compared to arginine-peptides [53]. Except for the broad ACPA response measured by Anti-CCP2, reactivities towards cit-vimentin and alpha-enolase-1 are particularly associated with HLA-SE [57, 154].

The association of ACPA-positive RA and any HLA-SE is strong with odds ratio (OR) of 6 and even higher OR when carrying two HLA-SE alleles [150, 155]. Even though the HLA-SE prevalence in ACPA-positive RA patients is increased, the sole presence of HLA-SE is not sufficient, as far as a large majority of the general population never developing RA also carry SE alleles (table 2) [156].

Table 2: Prevalence of number of HLA-SE alleles in Sweden

	General population (N=2,876 ¹)	ACPA-negative RA (N=1,281 ¹)	ACPA-positive RA (N=2,144 ¹)
No HLA-SE allele	48%	46%	15%
One HLA-SE allele	43%	45%	52%
One HLA-SE *04:01 or *04:04	27%	24%	41%
Two HLA-SE alleles	9%	9%	33%
Two HLA-SE *04:01 and/or *04:04	3%	3%	13%
One or two HLA-SE	52%	54%	85%

Data from epidemiological investigations in rheumatoid arthritis (EIRA), borrowed, with permission from Leonid Padyukov¹. Data from 4-digit typing was available in 1,822 controls; 921 ACPA-negative RA and 1,394 ACPA-positive RA patients.

More recent studies extending our understanding of the HLA DRB1 allele association, showed that amino acid at position 11, 71 and 74 and the amino acid risk haplotypes (at these positions: valine (V)-lysine (K)-alanine (A) (VKA); valine-arginine(R)-alanine (VRA) or leucine-arginine-alanine (LRA) are particularly associated with susceptibility to ACPA-positive RA [157, 158]. Across the HLA-alleles, there is a broad linkage disequilibrium and among the known HLA-SE alleles, HLA-allele *04 is linked to valine on position 11, HLA-allele *01 is linked to arginine on position 71 and alanine on position 74. Raychaudhuri et al [157] also found association of ACPA-positive RA with HLA-B allele at amino acid position 9 and with HLA-DPB1 allele at amino acid position 9, both within peptide binding groove of HLA-I respectively HLA-II. Some of these described amino acid haplotypes (in particular VKA, VRA and LRA) appear to be associated with specific clinical traits in patients with arthritis, RF-negative and RF-positive RA, such as radiographic progression (dependent and independent of ACPA), mortality and response to anti-TNF therapy [158].

There are many more suggested genetic risk factors for RA located outside of HLA, but they are of less strength [159]. Among these, protein tyrosine phosphatase (PTPN22) risk gene allele and cytotoxic T-lymphocyte antigen-4 (CTLA4) risk gene allele are also associated with ACPA-positive RA [160, 161]. Both PTPN22 and CTLA4 are suggested to be involved in B- and T-cell regulation.

1.5.1.2 Environmental and other risk factors

Smoking is the best-established environmental risk factor for ACPA-positive RA. An increased frequency of ACPA-positive RA is observed among ever smokers compared to never smokers with an OR of 1.6-2 [156, 162-164]. An increased strength in the association is observed with increasing smoking intensity [165, 166]. The increased risk due to smoking seems to remain several years after smoking cessation [167]. The effect of smoking in RA pathogenesis is suggested to be mediated through induction of posttranslational modifications of proteins in lung, possibly by inducing PAD (peptidylarginine deiminase) expression and citrullination of proteins that could activate an immune response and B-cells [149, 168-170]. In individuals both carrying HLA-SE and being exposed to smoking an additive interaction effect have been observed with maximum increased risk in individuals with two HLA-SE alleles and most heavy smoking (OR of 38) [149, 162]. The amino acid defined HLA-DRB1 risk haplotypes have shown similar additive interaction with smoking and susceptibility to ACPA-positive RA [165]. Silica is another airway exposure that has been shown to be associated with risk for ACPA-positive RA [171]. Other suggested risk factors for ACPA-positive RA are infections such as infection/colonization with *Porphyromonas gingivalis* [172], female gender and age [1], disadvantaged social economic status [173], no alcohol intake [174] and being overweight [175].

1.5.1.3 Familial risk factor and heritability

Familial background has been shown to be a factor with relatively great influence on the risk of developing RA, compared to other identified risk factors. However, it is important to stress that RA affects families only sporadically, with a minority of RA patients having first-degree relatives with RA (7-12%) [176]. Altogether, individuals with first degree relatives (parent, siblings, children) (FDR) or second-degree (half siblings, grandparents) relatives with RA have an increased risk of RA with OR and hazard ratio (HR) in the range of 2-4 [177-179]. Familial risk increase with a) increasing numbers of FDR with RA [177, 179], b) presence of ACPA-positive RA and c) disease onset before the age of 40 [177]. The relative importance of known environmental risk factors (such as smoking) appears to be small [176, 179].

Heritability (the relative influence of genetic factors for disease susceptibility in a population compared to the influence of environmental factors) for RA has been estimated in twin studies [180-183] with twin methodology and in case-control studies with analysis of similarities among siblings/families [177] or with GWAS kinship analysis [184, 185] (table 3).

Table 3. Heritability estimates for RA from twin and non-twin cohorts

Author (Year) Country		Terao (2016) Japan	Svendsen (2013) Denmark	McGregor (2000) UK	McGregor (2000) Finland	Woude (2009) UK	Frisell (2013) Sweden	Stahl (2012) UK, Sweden, USA, Canada	Lee (2015) UK, Sweden, USA, Canada, Netherlands, France
Details, cohort.		Twins recruited from RA cohorts (IORRA & KURAMA, N= 7,550).	National population based twin cohort.	Twins recruited via media and from hospital outpatient register.	National twin cohort. Sickness insurance register (up to year 1982).	Selected from UK McGregor (2000).	National population based case-control cohort.	WTCCC; EIRA; NARAC I-II; BRASS; Canada. case-control cohort.	Okada. WTCCC; EIRA; NARAC I-II; BRASS; Canada. case-control cohort.
Number patients (RF+/ ACPA+)		RA twins: 86 (78%/78%)	RA twins: 162 (80%/77%)	RA twins: 221 (85%/.)	RA twins: 261 (./.)	RA twins: 161 ¹ (./78%)	RA cases: 90,372 (78% ² /57%) ²	RA cases: 5,485 (100%/100%) ⁴	RA cases: 9,261 (87% / 87%) ⁶
Number individuals no-RA /controls		No-RA twins: 81	No-RA twins: 45,280	No-RA twins: 185	No-RA twins: 26,337	No-RA twins: 148 ¹	No-RA controls: 451,860	Controls: 22,621	Controls: 26,737
Methods		ACE/ AE- model	ACE model	Pop-prev. AE-model	AE-model	Pop-prev. AE-model	Sibling/ family similarities	GWAS analysis, common SNPs and kinship.	GWAS analysis, common SNPs and kinship.
Heritability (95%CI)	RA	56-62%	12% (0-76)	53% (40-65)	65% (50-77)	66% ¹ (44-75)	40%		
	RF+ RA			⁵			44% ²	45%	14-19% ⁶
	RF- RA						27% ²		0%
	ACPA + RA					68% ¹ (55-79)	50% ²	45%	14-19% ⁶
	ACPA- RA					66% ¹ (21-82)	20% ²		0%

¹Due to unclear selection and changes in the estimates (compared to the original cohort), this study is excluded from further interpretations. ²Limited data on RF-status (65% of cases had data) and ACPA-status (4% of cases had data). ³[186, 187]. ⁴[188]. ⁵Analyzed RF-positivity and bone erosions as markers for disease severity and state 'no quantitative genetic contribution to disease differed across disease severity groups'. ⁶Seropositive RA was defined as ACPA+ or RF+ or unknown status. Abbreviations: Institute of Rheumatology, Rheumatoid Arthritis (IORRA); Kyoto University Rheumatoid Arthritis Management Alliance (KURAMA); Wellcome Trust Case Control Consortium (WTCCC); North American Rheumatoid Arthritis Consortium (NARAC); Epidemiological Investigation of Rheumatoid Arthritis (EIRA); Brigham Rheumatoid Arthritis Sequential Study (BRASS). Reported data (from other studies) about RA population prevalence (Pop-prev) was used for estimating heritability. ACE-/AE-model includes additive genetic factors (A), shared environmental factors (C) and unique environmental factors (E). Positive (+); negative (-).

The heritability estimates have been in the range of 12-65% (median 45%) with tendency of lowered estimates in cohorts including more RA twins/cases and no-RA twins/controls. Two large studies have estimated heritability for RF-positive RA specifically to 44-45% and ACPA-positive RA specifically to 45-50% [177, 184]. Another large study estimated seropositive RA (including both RF and ACPA-positive) to be 14-19% but they included patients with unknown seropositive status (14%) in the analysis [185].

1.5.2 Risk factors for ACPA development

1.5.2.1 Genetic factors

Several studies have failed to identify significant associations between ACPA-positivity and presence of genetic factors known to increase the risk of developing RA such as HLA-SE and PTPN22 [189-192]. In contrast; a study performed in a Canadian family cohort of North American Native people with doubled risk of RA, ACPA positivity among relatives was significant associated with HLA-SE and HLA-DRB1*0901 allele [193, 194]. However, an important limitation to all these particular studies is the low number of analyzed ACPA-positive individuals (range 18-56).

1.5.2.2 Environmental and other risk factors

Preliminary results from a Dutch population-derived cohort (570 ACPA-positive out of 40,136 studied individuals aged 18-92 year) suggest a significant association between ACPA-positivity and smoking [195]. No significant association between ACPA-positivity and smoking but a trend were reported in one Japanese population-derived cohort (167 ACPA-positive out of 9,804 studied individuals, aged 30-75 year)[190] and two smaller family cohorts [191, 192].

Preliminary results from a Dutch population-derived cohort also suggest a significant association between ACPA-positivity and female sex in contrast to the Japanese population-derived cohort and a small family cohort [189, 190]. In the Japanese population-derived cohort Terao et al (2014) instead reported significant association between ACPA-positivity, increased CRP and increasing age.

1.5.2.3 Familial risk factor

The prevalence of IgG ACPA have been normal or slightly raised in many family cohorts (median 2.4%, range 1.2-6) [189, 192, 196-199] and clearly increased in multicase-RA-affected family cohorts (>1 FDR with RA) (median 18%, range 17.2-22) [191, 193, 197])

compared to what to expect (1-2%) in the general population [190, 200]. The detected ACPA in relatives is typically in lower titers, fewer isotypes and with fewer ACPA reactivities compared to their RA probands [191, 193, 201, 202]. At present the information regarding different ACPA reactivities is limited, but there are reports of relatively high prevalence of anti-cit-vimentin antibodies among FDRs (12-20%) [192, 197].

The relative role of genetic factors on the occurrence of ACPA was investigated in a Danish twin study including monozygotic (MZ) and dizygotic (DZ) twin pairs discordant for RA (N= 78 twin pairs)[203]. The study showed that MZ healthy twins with an ACPA-positive RA co-twin had increased risk for ACPA-positivity compared to DZ healthy twins, which suggest an overall susceptibility for ACPA-positivity driven by genetic factors.

1.5.3 Risk factors for transition from unclassifiable complaints to arthritis and RA

Studies of the phase of symptoms without arthritis typically investigate cohorts of individuals seeking health care and being referred to rheumatologist. Several interesting reports from a large Amsterdam Netherland cohort of individuals with arthralgia and autoantibodies (RF or ACPA) (N=374, 68% ACPA-positive, follow up time in median 32 months, 35% developing arthritis) have given details about risk factors associated with the transition from symptoms without arthritis to arthritis and/or RA. The dominant factor associated with risk for arthritis development in patients with autoantibodies and arthralgia is ACPA-positivity [68, 140, 204]. Having a FDR with RA, high titers of either ACPA or RF and not drinking alcohol are additional associated factors to arthritis development.

In a recent study of ACPA-positive individuals with musculoskeletal symptoms (n=100) from Leeds, UK; physician-assessed tenderness of small joints, presence of morning stiffness, high levels of RF or ACPA, presence of HLA-SE and ultrasound findings of power doppler signals were associated with arthritis development (50% developed arthritis after median 8 months (range 0.1-52)). Interestingly, no arthritis development was found in the ACPA-positive individuals lacking any of these factors, but this subgroup was very small (n=5) [142].

In a small arthralgia cohort (36 ACPA-positive out of a total of 55 individuals; 15 developed arthritis) smoking and overweight, but not ACPA positivity, was associated with arthritis development [141], but this observation was not confirmed in others studies [140, 142]. Later, a higher number of detectable ACPA reactivities was reported among individuals developing arthritis as compared to those not developing arthritis [205].

1.5.4 Risk factors for transition from unclassified arthritis to RA

Most of the patients presenting at the rheumatologist with detectable arthritis are easily classified in different diagnosis categories but a minority will not fulfill any classification/diagnosis criteria and will therefore be considered to have unclassified arthritis. Patients with unclassified arthritis and symptom duration <2 years have been investigated in the Leiden Early Arthritis Clinic (EAC) prospective population-based cohort. One year after follow up, about one third had progressed to RA and only a few percent of the patients progressed to RA during the further follow up (n= 570; ACPA-positive 21%; mean follow up 8 years) [206]. In the EAC cohort, risk factors for RA development were older age, female sex, family history of RA, increased CRP and ESR, and ACPA and RF [206, 207]. Unclassified inflammatory polyarthritis (IP) have been investigated in an English cohort (Norfolk), including patients with two or more swollen joints of at least four weeks duration (about 30% ACPA-positive). Alcohol use and no-manual work were lifestyle factors associated with decreased risk of developing ACPA- or RF-positive IP using prospectively collected data [208]. Severe disease outcomes such as functional disability and radiological destructions were associated with RF-positivity, increased CRP and health assessment questionnaire score (HAQ) [209].

1.6 TREATMENT CONSIDERATIONS IN RA

Treatment of RA aim primary to decrease disease activity, block cartilage and bone destruction and inhibit progression of comorbidities. There are several available DMARD, synthetic, small-molecules and biological agents, and new are emerging. The current EULAR (European League Against Rheumatism) recommendations for RA treatment suggest that a) therapy with DMARDs should be started as soon as the diagnosis of RA is made; b) MTX should be part of the first treatment strategy in patients with active RA c) in DMARD-naïve patients monotherapy or combination therapy of other synthetic(s) DMARDs should be used and that d) low-dose glucocorticoids should be considered as part of the initial treatment strategy (in combination with DMARDs) [210].

Response to therapy is highly variable in RA and several factors for persistent or erosive disease (high numbers of swollen joints, high levels of ESR/CRP, occurrence of RF and/or ACPA, presence of bone erosions and extraarticular disease) have been suggested [211, 212]. These factors are used to guide therapy today and their presences require a more aggressive therapeutic approach, despite not being clearly correlated with response to treatment. A lot of

effort has been put into development of new therapeutic biomarkers but a large majority of these efforts have failed and we still lack biomarkers that would specif

ically guide the use of current available drugs in different clinical settings in order to avoid both treatment failure and unnecessary drug overuse. For the purpose of this thesis, I will briefly discuss treatment with Methotrexate (MTX).

The mechanism of action for MTX seems to be related to dosage. In RA the dosage is in a low range compared to usage as anti-cancer therapy. Beside a role of MTX as purine synthesis antagonist inhibiting folate-dependent enzymes and DNA, RNA and protein synthesis the primary anti-inflammatory effect seen in RA is suggested to be adenosine-mediated. The active MTX metabolite inhibits enzymes that promote extra-cellular release of adenosine [213, 214]. Adenosine mediates a large array of responses to stabilize homeostasis. Adenosine receptors are expressed by immune cells and are responsive to the modulation of adenosine, which for example leads to changes in cytokine production by macrophages (less TNF and increased IL-10) and neutrophils; and effect T-effector-cell proliferation [215]. The effect by MTX seems to be more favorable compared to several other sDMARD [216]. The absolute treatment benefit with MTX, dosage varying 7.5-25 mg, compared to placebo is in 15-20% using ACR response definitions in patients with longstanding RA who previously failed other sDMARD (52 weeks in randomized and clinical control trials trials) [217]. However, there is an important inter-individual variation in response to MTX; about one third of patients have good response (using EULAR criteria for definitions), and about one fourth do not respond to MTX at all [218, 219]. In clinical routine MTX is often associated with adjuvant drugs such as glucocorticoid and NSAID (nonsteroidal anti-inflammatory drug), which aim to rapidly control for symptoms of the disease in a short time perspective. Glucocorticoid decreases bone destruction and has effect on disease activity and symptom relief and improve therapy response to MTX [220]. Glucocorticoid is a group of steroid hormones naturally produced in the adrenal cortex with multiple effects. Glucocorticoid can bind intracellular receptors and annexin-1 might be a mediator of the anti-inflammatory effects [221]. It is today not possible to predict MTX response, but male sex, smoking and high disease activity have been suggested to be clinical factors influencing MTX treatment response [219, 222-224]. No consistent association of MTX response with either antibody status or presences of genetic risk factors has been found [218, 225, 226].

As knowledge of how RA develops increase and with the emerging possibilities of assessing individual risk for future RA, a need for inhibiting and preventive treatments becomes apparent. Today's available treatments for RA target established pathological processes, but

future therapeutic strategies will probably be based on emerging pathogenic knowledge of the earlier processes.

2 AIMS OF THE THESIS

The overall aim of this thesis is to study anti-citrullinated protein antibodies (ACPA) as part of the pathogenesis of RA.

More specifically the aims of this thesis are:

- To investigate ACPA, characteristics of ACPA, associated risk factors and heritability of ACPA in a population-derived cohort among individuals without RA and patients with RA.
- To investigate the characteristics of ACPA, association between ACPA and bone destruction in early-untreated RA.
- To investigate Fc-glycosylation patterns of serum antibodies in early-untreated RA.

3 METHODS: HOW TO MEASURE AND STUDY ACPA AND RA?

In the following sections I will describe the main features of the cohorts and methods used in the articles and discuss some methodological concerns regarding these. Further details about the methods can also be found in each paper respectively.

3.1 RESEARCH COHORTS, DATA SOURCES AND REGISTER

3.1.1.1 Swedish twin register (STR)

In study I-II we used a research cohort called Twingene derived from the national Swedish Twin Registry (STR) (see figure 4 for overview of study recruitment and data collection). STR is one of the world largest twin registers and started in the late 1950ies. STR compiles in principle all twins born in Sweden between 1886 and onward [227] and the coverage of birth cohort 1925-1958 is very good [228]. Birth cohorts of twins have regularly been contacted and asked to participate and answer questionnaire about health and zygosity.

3.1.1.2 Screening across lifespan study (SALT)

Twins from the Swedish Twin Registry born 1886-1958 were invited to participate in the Screening across lifespan study (SALT) during year 1998-2002. Participating twins were interviewed by telephone using a standard health questionnaire. Response rates were 65% for twins born 1886-1925 and 74% for twins born 1926-1958 [227]. Smoking habits in the Twingene cohort (see below) were evaluated using data from the SALT study.

3.1.1.3 Twingene-study

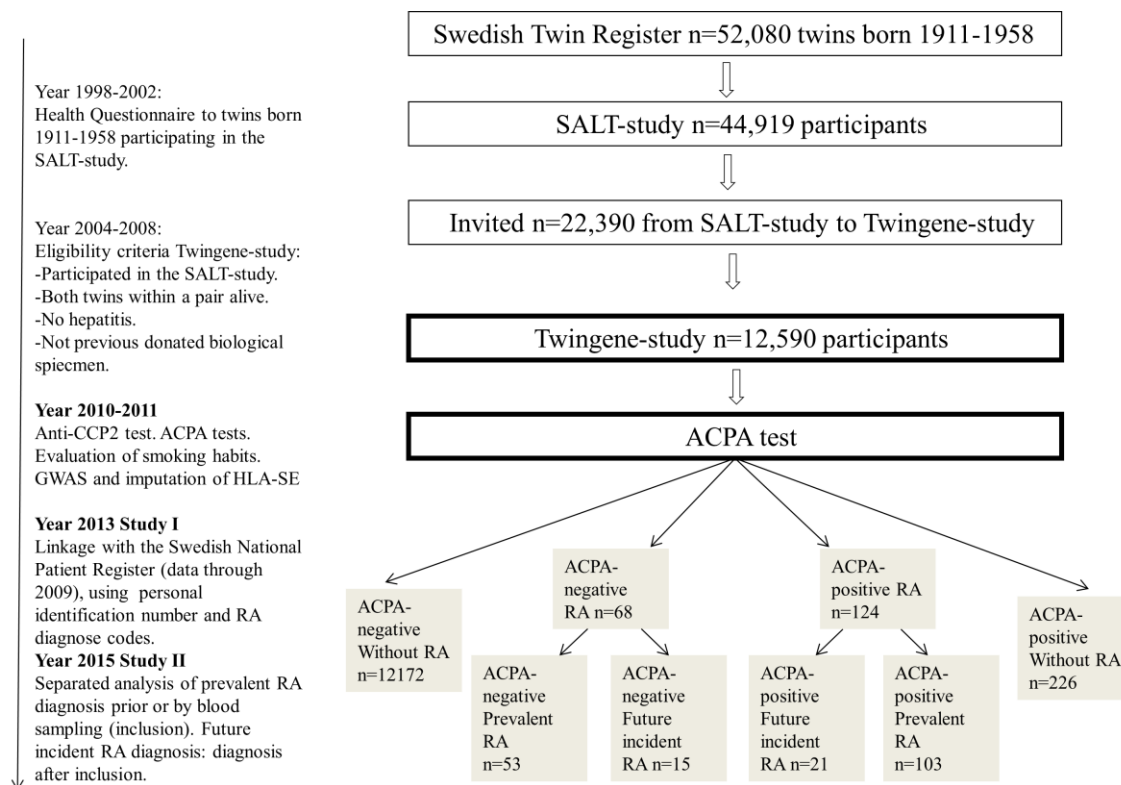
Twins that participated in the earlier SALT study and had a living co-twin were asked to participate in the Twingene-study year 2004-2008 (N= 22,390) [229]. Twins that participated in SALT and donated blood in another twin study and had a record of hepatitis or had declined further participation in STR studies were excluded from participation [230]. Response rate was 56% (pair-wise response rate was 45%) and resulted in enrollment of 10,004 twins in complete pairs and 2,590 twins without their co-twin (55% female; 28% MZ pairs, 72 % DZ pairs).

Information about the Twingene-study with the overall aim to investigate complex diseases and a questionnaire about health status were sent by mail, starting with the oldest age-groups. Consenting participants were then asked to donate blood in outpatient clinics throughout the

country [229]. Donated blood was sent by mail to Karolinska Institutet Biobank and Karolinska University hospital laboratory. At arrival samples were immediately analyzed for serum analytes such as CRP. Serum was also aliquoted and stored, while DNA was extracted from whole blood and subsequently stored. DNA was analyzed genome-wide for selected single nucleotide polymorphisms (SNP) and data for GWAS and imputations of genotypes were provided.

Earlier, zygosity was determined by self-reported childhood resemblance in the SALT study. This is a method that has been validated several times with DNA testing and has $\geq 95\%$ accuracy [231]. Subsequently GWAS analysis of all DZ twins and one MZ twin (within a pair) could once again validate this as a high accuracy method.

Figure 4. Overview of study recruitment and data collection



3.1.1.4 The National Patient Register

We used data from the Swedish National Patient Register (NPR) from the National Board of Health and Welfare in study I-II. NPR covers all public care and contains information about hospital discharges since 1964, with gradually increase coverage of counties and complete national coverage since 1987 [232]. Information from visits at outpatient non-primary care specialist clinics has been included since 2001. NPR have almost absolute completeness with

regards to the hospital discharges and 75-99% coverage of somatic diagnosis of outpatients [233]. The correctness of RA diagnosis in NPR from hospital discharges and outpatient care has been validated in studies using medical records and ACR 1987 criteria for RA [4]. More than 1500 patients have been included in these studies and a correctness of about 90% have been found [234-236]. In study I-II we used the validated diagnosis codes and considered a person to have RA if an RA diagnosis was listed in the NPR at least once in 2009 or earlier. Personal identification numbers were used as keys when linking between Twingene and NPR.

3.1.1.5 Epidemiological investigation for rheumatoid arthritis (EIRA) and the Swedish rheumatology quality register (SRQ)

In study III-IV we investigated a clinical cohort of early-untreated RA patients deriving from Karolinska University Hospital in Solna. A nationwide patient cohort originated from EIRA with SRQ data [219] was used to identify these patients in Solna. The patients included were newly diagnosed, had symptom duration of <1 year and the vast majority were DMARD naïve. In study III an additional 1,116 (none Solna) patients were selected from the EIRA study with available data on standard radiographs and serum ACPA status as validation cohort.

Epidemiological investigation for rheumatoid arthritis (EIRA) is a population-based case controls study of incidence cases of RA, started in 1996. The Swedish rheumatology quality register was initiated 1995 and includes patients with RA. At inclusion information about ACR 1987 criteria, onset of symptoms and date of diagnosis are collected in the register. Additional information from clinical visits is subsequently added, such as current treatments, CRP, ESR, HAQ and DAS28.

3.1.1.6 Rheumatology unit biobank at Karolinska Institutet

Since the 1990ies the rheumatology unit at Karolinska University Hospital Solna has collected serum samples from newly diagnosed RA patients for research purposes. Samples have been taken at initiation of treatments and at follow up and stored in a biobank. Information about personal identification number and preliminary diagnosis is linked to the biological specimens as well as sample handling. The biobank also include population controls. In study III we gathered a cohort of patients with early-untreated RA, from Karolinska University Hospital Solna, with available serum samples collected at MTX initiation and at 3 month follow up (N= 183). In addition, synovial tissue obtained during

arthroscopy from 15 patients with early-untreated RA (7 of the patients that donated serum samples) was analyzed. In study IV, only a selection of patients in study III was analyzed (N= 59/183) due to limitations in analysis capacity. We randomly selected an equal number of patients among the three EULAR response groups (none response/moderate response/good response). We additionally analyzed 11 population controls from the EIRA study, matched to the cases by age, sex and sample storage information.

3.2 MEASUREMENT OF SERUM PROTEINS AND GLYCANS ON ANTIBODIES

3.2.1 Enzyme linked immunosorbent assay

Anti-CCP2, ACPA reactivities and RANKL proteins were analyzed by ELISAs. For analysis of Anti-CCP2 antibodies we used the commercial available ELISA kit CCPlus Immunoscan, (Malmö, Sweden) (figure 5) (study I-IV). For analysis of ACPA reactivities toward specific cit-peptides we used established in house ELISA (study I-IV). We analyzed antibody reactivities toward cit-alpha-enolase-1, type II collagen (not study III-IV), fibrinogen and vimentin and particular epitopes (table 4).

Table 4: Peptides used in ELISA for measuring different ACPA reactivities

Abbreviations: cit: citrullinated, eno: enolase, vim: anti-cit-vimentin, col: anti-cit-collagen

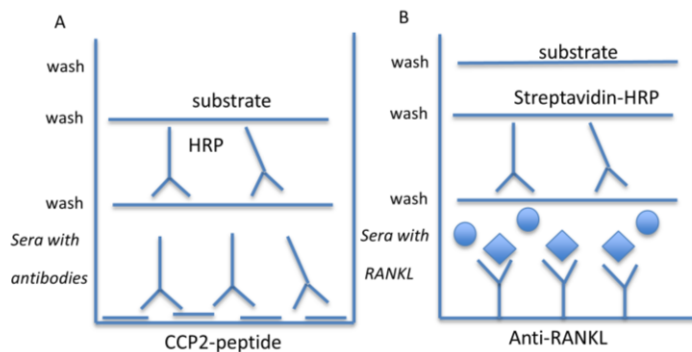
Name	Peptide	Protein	Amino acids; citrullines at position
Anti-cit enolase	CEP-1	α -Enolase-1	5-21; 9 & 15
Anti-cit vimentin	Vim 60-75	Vimentin	60-75; 64, 69 & 71
Anti-cit fibrinogen	Fib α 563-583	Fibrinogen α -chain	563-583; 573
Anti-cit collagen	CII C1	Type II collagen	359-369; 360 & 365

Native forms of the same cit-peptides were used as control peptides. All samples were run in duplicates and compared with standard curves. These proteins and the selected epitopes are considered well-established targets for ACPA antibodies in RA patients as they occur locally in the joint. Further, the targeted cit-epitope(s) have been mapped and the antibodies have been repeatedly found to occur in large RA cohorts [55, 57, 58, 237-239]. The cut-offs for the different ACPA reactivities were established using the 98th percentile in measured sera from 152 population controls. All ACPA ELISAs were semi-quantitative ELISAs, which generated relative concentrations within the range of detection. For Anti-CCP2 ELISA the lower detection range coincide with the set cut-off for normal-abnormal/negative-positive

concentration. Therefore, we were not able to analyze below the cut-off. In retrospective, it would have been an advantage to also gather more data by modifying the protocol and adding an extra standard, below the set cut-off. In study III, the Anti-CCP levels above the highest standard (>3200 AU/ml) were reassessed after appropriate dilution.

For study III and the analysis of RANKL we used the commercial available ELISA kit sRANKL Biovendor, Brno, Czech Republic, that measures total (bound and unbound) soluble RANKL. Paired samples (baseline and follow-up) were run at the same assay. In parallel to analyzing all patient data, we did a separate analysis of RF-negative patients and thereby eliminated the bias of RF interference. Data on RF in study III-IV was gathered from medical records and reported test results from Karolinska University Hospital Laboratory.

Figure 5: ELISAs for detecting A) Anti-CCP2 B) RANKL serum protein.



Abbreviation: horseradish peroxidase (HRP).

3.2.1.1 Other immunoassays

In study II we analyzed gathered data about CRP. The CRP level had been analyzed at inclusion by a near infrared particle immunoassay rate method using automated equipment at Karolinska University Hospital [240]. In study III serum concentrations of CRP, IL-6, and TNF receptor type 1 (TNF-RI) were measured by multiplex sandwich immunoassay, with biomarker-specific capture-antibodies printed to specific locations in each well and detection-antibodies labeled with electrochemiluminescent tags (Sector Imager 6000, Meso Scale Discovery, Gaithersburg, MD, USA) [241]. Information about CRP was also used in study IV.

3.2.2 Immunohistochemistry

Synovial expression of RANKL in study III was detected by immunohistochemistry staining, using a monoclonal anti-human RANKL detection-antibody (12A668, Abcam, Cambridge, UK) [17, 242]. The RANKL expression level was evaluated using computer-assisted image analysis and expressed as the percentage of the total tissue area that stained positive.

3.2.3 Mass spectrometry and characterizing Fc-glycans on antibodies

In study IV, we characterized the Fc-glycopeptides within the pool of other extracted peptides that were obtained using a regular proteomic analysis approach [243]. Briefly 10 µg of serum proteins were cleaved with trypsin. Peptide analysis was performed via liquid chromatographic (LC) peptide separation (ReproSil-Pur-LC-system (Easy-nLC II, Thermo Fisher) and peptide fragmentation and sequencing on a QExactive orbitrap mass spectrometer (MS) (Thermo Fisher Scientific). Mass spectra lists were extracted and searched against databases and IgG proteins were quantified. IgG₁ and IgG₂ Fc-glycopeptides were identified by their retention times and mono-isotopic masses [63, 244]. Glycans were quantified using a similar approach as with the proteins [245]. In total 12 IgG₁ and 7 IgG₂ Fc-glycopeptides were found on quantified levels. Glycan abundances were normalized to total content (100%) of Fc-glycosylated IgG₁ peptides and total content (100%) of Fc-glycosylated IgG₂ peptides.

3.3 MEASUREMENT OF DISEASE ACTIVITY AND RESPONSE TO TREATMENT

Disease activity in diagnosed RA can be measured in several ways. One common way is using a composite measurement called *disease activity score 28 joints* (DAS28) (figure 6) that is adjusted to include the typical involved joints [246]. This measurement was used in study III-IV. DAS28 was developed to standardize clinical judgment of high contra low disease activity, originally defined as the time point when the rheumatologist decided, that the patient should start respectively stop DMARD treatment [247]. Later, the DAS28 score was suggested to be categorized as high, moderate and low disease activity, which originally also was based on rheumatologist treatment decisions [248, 249]. The moderate category represented disease activity with discordant clinical treatment decisions and a statistical no significant decrease compared to high disease activity (calculated from repeated measurements and measurement error).

Figure 6: Formula defining DAS28-ESR and included parameters

$$\text{DAS28} = 0.56\sqrt{\text{TJC28}} + 0.28\sqrt{\text{SJC28}} + 0.70 \cdot \ln(\text{ESR}) + 0.014 \cdot \text{VAS GH (Pat)}$$

Measurements of changes of disease activity are important to evaluate the effect of treatment. This is often done by using a composite measurement (similar to DAS28) of change. EULAR response criteria (table 5) and ACR response criteria are commonly used [250, 251]. In study IV we used the EULAR response criteria as all information necessary for the ACR response criteria was not systematically collected in the utilized clinical derived cohort. An important implication to the use of EULAR and ACR response criteria as golden standards for defining response when doing research about predictive factors for response is that both of these include measurements of acute phase reactants (CRP or ESR).

Table 5: EULAR response criteria

DAS28 improvement →	>1.2	>0.6 and ≤1.2	≤0.6
Present DAS28 ↓			
≤3.2	Good response	<i>Moderate response</i>	No response
> 3.2 and ≤5.1	<i>Moderate response</i>	<i>Moderate response</i>	No response
> 5.1	<i>Moderate response</i>	No response	No response

3.3.1 Bone destruction

In study III we gathered data on bone destruction at diagnosis in the investigated cohort and in a validation cohort. In the investigated cohort we gathered data from medical records about presence of bone erosions (defined as at least one) on radiographs of hands and feet reported by rheumatologist and/or radiologist. In the validation cohort we used information from SRQ about RA-typical bone changes according to the 1987 RA criteria reported by rheumatologist [4]. Accordingly, we were limited to analyze bone destruction as a binary variable – present or not present.

3.4 STATISTICAL ANALYSIS

Pearson's chi-square test, Wilcoxon rank sum test and Wilcoxon signed rank test were used for independent and paired comparisons as appropriate. Normally distributed continuous

variables were compared with Student's t-test respectively Student's paired t-test. Statistical analyses were conducted using SAS 9.1-9.3 (SAS Institute Inc., Cary, NC, USA) at a significance level of 0.05.

3.4.1 Study I

We used a binomial logistic regression model to analyze relationships between ACPA-positivity and different exposures and adjusted for clustering of data, as we analyzed a twin cohort. Estimates of associations were presented as odds ratio (OR: odds for the outcome if exposed compared with the odds for the outcome if not unexposed). Heritability for ACPA-positivity and RA was estimated by using structural equation models accounting for the contribution of additive genetic, shared environmental, and non-shared environmental factors (the so-called ACE model) or additive genetic, dominant genetic and non-shared environmental factors (the so-called ADE model). Heritability is the proportion of variance in liability, to a certain phenotype, which is due to variance in genetic factors. By comparing the liability in monozygotic twins, assuming that MZ share all genetic factors, with the liability in DZ twin, assuming that DZ only share half of the genetic factors, the relative role of genetic factors and environmental factors can be estimated. Underlying assumptions when doing modeling of heritability is that there is no gene-gene interaction and no gene-environment interaction of importance. Heritability estimations were conducted in the statistical package Mx.

3.4.2 Study II

The analysis focused on evaluating diagnostic accuracy for Anti-CCP2 test. Thus we calculated sensitivity, specificity, positive and negative predictive values (PPV/NPV), and positive and negative likelihood ratio (PLR/NLR) for prevalent RA at inclusion by 2x2 tables. We used a log binominal regression model for estimating the relative risk (RR, the risk for outcome if exposed compared with the risk for the outcome if unexposed) of having prevalent RA at inclusion if testing positive for Anti-CCP2.

3.4.3 Study III

In order to investigate the association between RANKL concentration (logarithm-transformed concentration) and ACPA, univariate linear regression models and multivariate regression models were used (including significant predictors from univariate analyses).

3.4.4 Study IV

Multivariate statistical modeling was used to test the correlation between baseline and changes in disease characteristics, IgG isotype, IgG Fc-glycans; and EULAR response.

Orthogonal projections to latent structures – discriminate analysis (OPLS-DA) were performed using SIMCA 13.0 (Umetrics, Umeå, Sweden). A receiver operating characteristic (ROC) curve analysis was performed to establish optimal cut off for suggested predictive factor.

4 RESULTS AND DISCUSSION

In this section, I will present results from study I-IV and discuss them in the light of previous and recent findings in other studies.

4.1 SCREENING A LARGE TWIN RESEARCH COHORT FOR STUDYING ACPA AND THE DEVELOPMENT OF RA

When we initiated these studies of ACPA, the available knowledge about occurrence of ACPA in a population setting was limited. In a population-setting, one might expect to find all phases and combinations of phases leading up to RA, ranging from individuals being or not being at risk of developing RA, up to individuals with actual full-blown, diagnosed and treated RA. ACPA had previously been studied in selected cohorts of individuals without arthritis or RA, but with an elevated risk of developing arthritis (e.g. FDR of RA patients) [189, 191-193, 197] or in selected patient cohorts, such as patients with arthralgia [140, 141] and patients with RA in which serum samples antedating the RA diagnosis were analyzed [58, 62, 126, 127, 129, 131, 132] or RA cohorts [57, 166, 252]. Hence, our first approach to study ACPA was to test a large population-derived twin cohort (N=12,590, called Twingene) by using the clinically established Anti-CCP2 test for occurrence of ACPA. By applying information from the Swedish National Patient Register about RA diagnosis (though year 2009), we could then separate ACPA-positive individuals without RA from ACPA-positive patients with RA. We identified a large group of the former in comparison to previous cohorts.

We found ACPA present in patients with RA at prevalence 65%, and in individuals without RA at prevalence 1.8% (study I). The prevalence in RA patients was in accordance with previous studies in RA cohorts [57, 166, 252]. The prevalence in individuals without RA was corresponding with previous and recent reports in population-derived studies of healthy and no-RA individuals [190, 200].

The large population-derived twin cohort (N=12,590) we tested, originated from the Swedish twin register and all participating twins have been included in at least one prior research study (see figure 4 in the methods section). This made us concerned about possible selection-effects on the participating twins in relation to the general Swedish population. With regards to the frequency of participating females, occurrence of HLA-SE and smoking, this was similar to the previously reported prevalence in the Swedish population [156, 253, 254]. The median age of the register participants was 64 years (1st-3rd quartile 60-70, range 48-93), which was a

result of restricted recruiting of twins born 1911 to 1958. Compared to national age-distribution (in the range 48-93 years) the age-group 61-70 year old was overrepresented in Twingene (29% versus 47%)[254]. This was a limitation in our power to analyze effect by age and to generalize our findings to younger and older cohorts. However, we don't think that participation in the study, for any of the age-groups, was driven by ACPA-status, since this was unknown for most participants at the time of inclusion and the information given to all participants was of general character describing the overall aim of studying complex inflammatory diseases (and thus not ACPA- or RA-specific). Another limitation has to do with the focus of ACPA and RA and the lack of information regarding presence of other autoimmune disease among the studied twins.

4.2 PRESENCE OF ACPA AND ESPECIALLY HIGH TITERS OF ANTI-CCP2 HAVE A HIGH DIAGNOSTIC ACCURACY FOR AN RA DIAGNOSIS IN A POPULATION SETTING.

In study II we investigate the relation between ACPA occurrence and RA diagnosis and estimated the diagnostic accuracy of the Anti-CCP2 test. We identified 156 (1.2%) individuals that had received a diagnosis of RA at the time of inclusion in the cohort, and were referred to as prevalent RA cases. During an overall median follow up of 37 months (1st-3rd quartile: 31-49), an additional number of 36 individuals (0.3%) received a first ever RA diagnosis (in median 21 month after inclusion (1st-3rd quartile: 8-27)) and were consequently categorized as future incident RA. Overall, our results regarding the prevalence and incidence of RA were similar to that expected in the general population of the same age [1, 2].

We estimated the relative risk (RR) for having prevalent RA for an individual testing positive for ACPA (using the Anti-CCP2 test) (Table 6). The RR for prevalent RA was 64 (95% CI: 46-88) if Anti-CCP2 test was positive and 94 (95% CI: 70-127) if using a high Anti-CCP2 cut-off (>3 times higher than the cut-off value). We are currently analyzing the risk for an ACPA-positive individual to develop future incident RA (Table 6-7) (data not published). Overall the estimated diagnostic accuracy (sensitivity, specificity, and RR) is similar for Anti-CCP2 test predicting prevalent RA and Anti-CCP2 test predicting future incident RA except positive predictive value. The positive predictive value (PPV) for Anti-CCP2 with regards to future incident RA was 8.5% (95% CI: 5.0-12.0), and 17.1% (95% CI: 9.9-24.4) for the high Anti-CCP2 cut-off. These estimates are low and similar to previous suggestions of 5-16% by Nielen et al (2004) and Rantapää Dalhqvist et al (2003), who used results about diagnostic

accuracy of Anti-CCP test in samples antedating RA onset and information regarding national incidence rate in Netherlands respectively Sweden [126, 127]. A recent Mexican prospective study in FDR to RA, reported higher PPV (60%, NPV: 99%) possibly as a result of elevated risk of developing arthritis (there was no information about inclusion of multicase families) and longer follow up (5 years) [198].

Table 6: Relative Risk for A) prevalent RA diagnosis at inclusion and B) future incident RA diagnosis; associated with ACPA positivity.

A)

Test	RR	(95% CI)
Anti-CCP2 test	67	(34-129)
High anti-CCP2 cut-off	114	(61-212)

B)

Test	RR	(95% CI)
Anti-CCP2 test	67	(34-129)
High anti-CCP2 cut-off	114	(61-212)

All estimates and confidence intervals (CI) were adjusted for sex, CI also adjusted for clustering of data. High Anti-CCP2 cut-off is titers >3x cut off.

With regards to RF-testing, data from a Danish population-derived cohort (mean age 50 year) show PPV of 5.5% for future RA (within 10 years, using an optimized cut off) and NPV of 99.6% [255]. .

The estimates about the diagnostic accuracy of Anti-CCP2 test for future incident RA are preliminary and further validation is required and underway to account for the uncertainty related to the actual date of disease onset that could be prior to the date of diagnosis. The median follow-up time from inclusion to diagnosis were quite short; 21 months (1st-3rd quartile: 8-27), but reasonable if comparing with data from arthralgia cohorts suggesting two year phase with symptoms before arthritis development [140, 142]. The uncertainty with regards to the actual onset will also affect the difference between the diagnostic accuracy for Anti-CCP2 and high Anti-CCP cut-off as it is known that Anti-CCP2 gradually increases during development of RA. With these limitations in mind, there are similarities with our estimates and the estimates in studies referred above. Overall, our results suggest that the Anti-CCP2 test (especially high titers of Anti-CCP2) is a good test to predict prevalent RA,

and to identify individuals at increased risk of developing RA in a population. The PPV for Anti-CCP2 with regards to future incident RA is low, but it will probably increase with increasing follow up time. The eventual additional value for the Anti-CCP2 test screening for earlier RA diagnosis in comparisons to accessible health care and rheumatology clinics is uncertain, unexplored and a question for future research.

Table 7: Diagnostic accuracy of ACPA test for A) prevalent RA at inclusion and B) future incident RA diagnosis

A)

Test	Result	RA	no RA	Sensitivity	Specificity	PPV	NPV
				(95%CI)			
Anti-CCP2 test	Positive	103	247	66.0%	98.0%	29.4%	99.6%
	Negative	53	12187	(58.6-73.5)	(97.8-98.3)	(24.7-34.2)	(99.5-99.7)
High Anti-CCP2 cut-off	Positive	97	105	62.2%	99.2%	48.0%	99.5%
	Negative	59	12329	(54.6-69.8)	(99.0-99.3)	(41.1-54.9)	(99.4-99.6)

B)

Test	Result	RA	no RA	Sensitivity	Specificity	PPV	NPV
				(95%CI)			
Anti-CCP2 test	Positive	21	226	58.3%	98.2%	8.5%	99.9%
	Negative	15	12172	(42.2-74.4)	(97.9-98.4)	(5.0-12.0)	(99.8-99.9)
High Anti-CCP2 cut-off	Positive	18	87	50.0%	99.3%	17.1%	99.9%
	Negative	18	12311	(33.7-66.3)	(99.2-99.5)	(9.9-24.4)	(99.8-99.9)

PPV and NPV denote positive and negative predictive value.

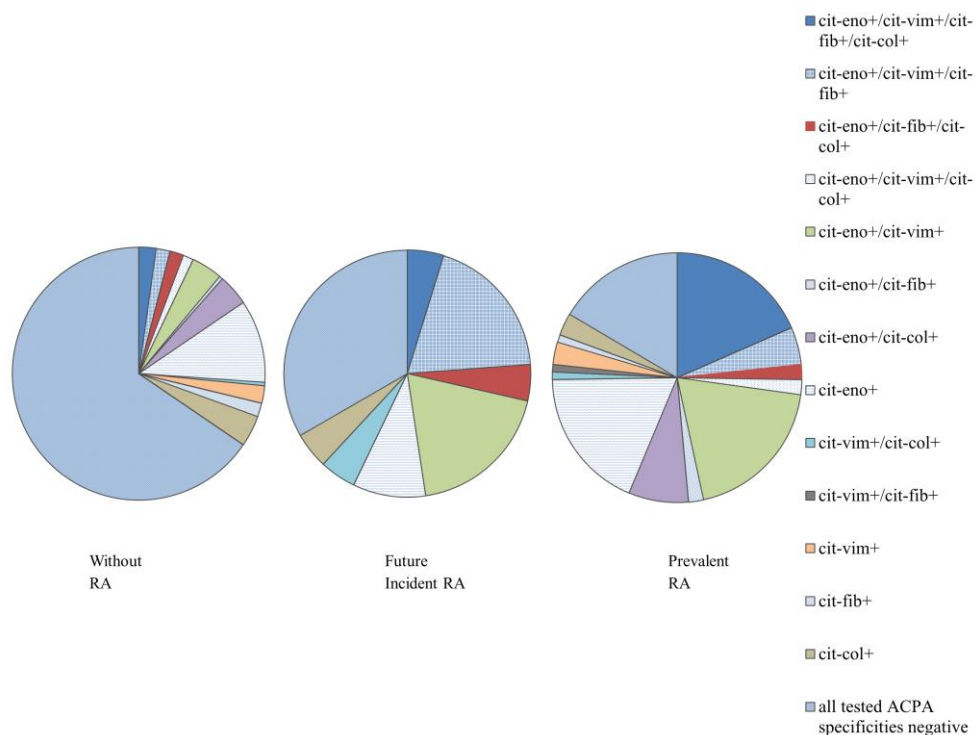
4.3 CONCENTRATION AND ACPA REACTIVITIES DIFFER IN INDIVIDUALS WITHOUT RA COMPARED WITH PATIENTS WITH RA

In ACPA-positive individuals without RA, Anti-CCP2 titers (51 AU/ml; 1st-3rd quartile: 34-149) were lower as compared to ACPA-positive individuals developing future incident RA (193 AU/ml; 1st-3rd quartile: 150-781) and ACPA-positive RA patients (508 AU/ml; 1st-3rd quartile: 194-1060) (study II).

In parallel, the number of recognized cit-peptides were different and a majority of the sera from ACPA-positive individuals without RA did not recognize any of the tested cit-peptides (median 0 (1st-3rd quartile 0-1)) in contrast to individuals who later developed future incident ACPA-positive RA (median 2 (1st-3rd quartile 0-3)) and patients with prevalent ACPA-positive RA (median 2 (1st-3rd quartile 1-3)). Low Anti-CCP2 titers in Pre-RA samples antedating the RA diagnosis with several years (in median 5 years) has also been reported in a study by Turesson et al (2011) [131]. The Anti-CCP2 concentration in these Pre-RA samples was in median 4 times the cut-off (ranging from 2-9 times) and are comparable with Anti-CCP2 concentrations in samples from individuals without RA in median 2 times the cut-off (ranging from 1.5-6 times). There were significant association with 'Low' titer ACPA (1-3 times the cut-off) and future incident RA (prevalence 8% (3/36)) or prevalent RA (prevalence 4% (6/156)) in the population-derived cohort (prevalence 1% (139/12398)), suggesting that low titer ACPA is also relevant for RA (but not as common as high titer).

In the group of ACPA-positive individuals without RA that recognized at least one of the tested cit-peptides (N=78) there were large numbers of different profiles of recognition of cit-peptides similar to the number of profiles found in ACPA-positive RA (figure 7). With regards to previous studies in RA, broad patterns of different profiles of recognized cit-peptides are characteristically for RA development and RA [56-58]. Anti-cit enolase was the most frequent occurring reactivity.

Figure 7: ACPA profile among ACPA (Anti-CCP2) positive individuals



4.4 HEAVY SMOKING AND HLA-SE ARE FACTORS ASSOCIATED WITH DEVELOPMENT OF ACPA

In study I, we investigated the role of smoking and HLA-SE for developing ACPA per se and compared with the influence these factors had on ACPA-positive RA. In study II we separately defined the group of ACPA-positive future incident RA and prevalent RA at inclusion and in the following section results from this phase will also be presented (unpublished data). Heavy smoking (>10 pack-years) was significantly associated with the presence of ACPAs without RA (OR: 1.4, 95% CI 1.1-1.9) and ACPA-positive RA (OR: 1.5, 95% CI 1.0-2.3) (table 8). Individuals with ACPAs without RA, individuals with ACPA-positive future RA and patients with ACPA-positive prevalent RA, were equally exposed to smoking. Our results regarding association with ACPA (in individuals without RA) and smoking are concordant with later reports from Japan (showing a trend of increased association with ACPA and heavy smoking) and the preliminary results from a Dutch cohort [190, 195].

Table 8: Association between ACPA and smoking

	ACPA-positive without RA (n=224)	ACPA-positive Future incident RA (n=21)	ACPA-positive Prevalent RA (n=103)	ACPA-positive RA (n=124)
Not smoking	Reference	Reference	Reference	Reference
Smoking				
OR	1.27	1.74	1.30	1.36
(95% CI)	(0.97-1.65)	(0.72-4.20)	(0.88-1.92)	(0.95-1.95)
P Value	0.09	0.22	0.19	0.09
≤10 pack years				
OR	1.10	0.72	1.29	1.20
(95% CI)	(0.75-1.59)	(0.15-3.40)	(0.77-2.17)	(0.74-1.97)
P Value	0.63	0.68	0.34	0.46
>10 pack years				
OR	1.42	2.68	1.32	1.52
(95% CI)	(1.05-1.93)	(1.08-6.65)	(0.83-2.07)	(1.01-2.27)
P Value	0.02	0.03	0.24	0.04

All comparisons are made using ACPA-negative individuals (n=12,173) as comparator group. Missing data on pack years from 552 individuals. Confidence intervals (CI) are adjusted due to clustering of twin data and sex. P values are estimated by Wald statistics and ChiSq.

HLA-SE was significantly associated with the presence of ACPA without RA (OR: 1.4, 95% CI 1.1-1.9) and ACPA-positive RA (OR: 7.2, 95% CI 4.0-13.2) (table 9). The association between HLA-SE and ACPA-positive individuals without RA was weaker than the association between HLA-SE and ACPA-positive RA, as ACPA-positive individuals without RA was less frequent carriers of HLA-SE than patients with ACPA-positive prevalent RA. There was also a trend of less frequent carriers of the HLA-SE alleles in ACPA-positive individuals without RA (60%) compared to ACPA-positive future incident RA (80%, p-value: 0.13).

The significant association between ACPA and HLA-SE in individuals without RA has not been reported previously. A few studies has been looking into this association, several reports trends of association but the overall fail of significant association is probably due to low number of observations [189-192].

Table 9: Association between ACPA and HLA-SE

	ACPA-positive without RA (n=190)	ACPA-positive Future Incident RA (n=15)	ACPA-positive Prevalent RA (n=103)	ACPA-positive RA (n=104)
No HLA-SE allele	Reference	Reference	Reference	Reference
Any HLA-SE				
OR	1.42	3.78	8.39	7.24
(95% CI)	(1.06-1.90)	(1.07-13.38)	(4.21-16.74)	(3.96-13.22)
P Value	0.02	0.04	<0.0001	<0.0001
One HLA-SE allele				
OR	1.25	3.89	6.23	5.65
(95% CI)	(0.91-1.71)	(1.07-14.15)	(3.06-12.71)	(3.03-10.52)
P Value	0.17	0.04	<0.0001	<0.001
Two HLA-SE alleles				
OR	2.13	3.27	17.46	13.91
(95% CI)	(1.41-3.22)	(0.55-19.62)	(8.27-36.85)	(7.15-27.08)
P Value	0.0003	0.19	<0.0001	<0.0001

All comparisons are made using ACPA-negative individuals (n=10,508) as comparator group. Confidence intervals (CI) are adjusted due to clustering of twin data. P values are estimated by Wald statistics and ChiSq.

A limitation in our study (which we share with other studies, and which concern all reported results (particular in study I and II)) is the cross-sectional nature of the data about ACPA-status, which was measured at only one time point. We do not know the time point for the emergence of ACPA, meaning that we lack information regarding time relationship for the smoking exposure. The information about the participants' smoking habits was collected

several years prior (prospectively) to inclusion into the Twingene cohort and donating blood. Regarding the exposure for HLA-SE we can assume exposure prior to ACPA occurrence, as HLA-SE is constant basal expressed from birth.

In a recent Japanese report, age was suggested a risk factor for developing ACPA (the cohort included age 18-92 years), in contrast to preliminary result in a Dutch population-derived cohort and a previous study of ACPA among patients without RA in a clinical setting [195, 256]. Other studies on the role of age for RF have also been conflicting [255, 257, 258]. In Twingene, the age-structure was a limiting factor, as about half of the included twins were within the age span 61-70 years, but there was a significant association between ACPA without RA and age when analyzed as a continuous variable or as a categorical variable (unpublished data), similar to data from the population-derived Japanese cohort.

In general terms, it has been suggested, that female sex influence the susceptibility for certain autoantibodies and we also found female sex significantly associated with ACPAs without RA, similar to the preliminary result in a Dutch cohort [195]. Female sex was not associated with ACPA in the Japanese cohort, which could have to do with differences in underlying environmental factors between the European and Japanese population and/or due to selection of the Japanese cohort in which individuals ‘possibly having autoimmune diseases’ were excluded [190].

Another relative strong risk factor for rheumatoid arthritis is having a first degree relative with RA [177]. In accordance with this study, we also found a significant association between RA and having a co-twin with RA in the Twingene cohort (unpublished data). However, if we investigated the role of family factor and risk for ACPA, we found only weak non-significant positive association. To further investigate the familial aggregation of RA and the probable familial aggregation of ACPA, which could be influenced by both shared environmental and shared genetic factors, we used another approach (study I) and analyzed the cohort with twin methodology.

4.5 ENVIRONMENTAL FACTORS ARE IMPORTANT FOR THE VARIABILITY IN ACPA STATUS

In study I we estimated the relative role of genetic and environmental factors for the variance in liability for ACPA-positivity and RA in a population. The estimated variance assigned to genetic factors was 10% (95% CI: 0-43) for ACPA without RA; 22.5% (95% CI: 0-45) for ACPA-positivity, 41% (95% CI: 0-54) for ACPA-positive RA and 39% (95% CI 0-66) for RA (irrespective of ACPA status). The estimated variance assigned to unique

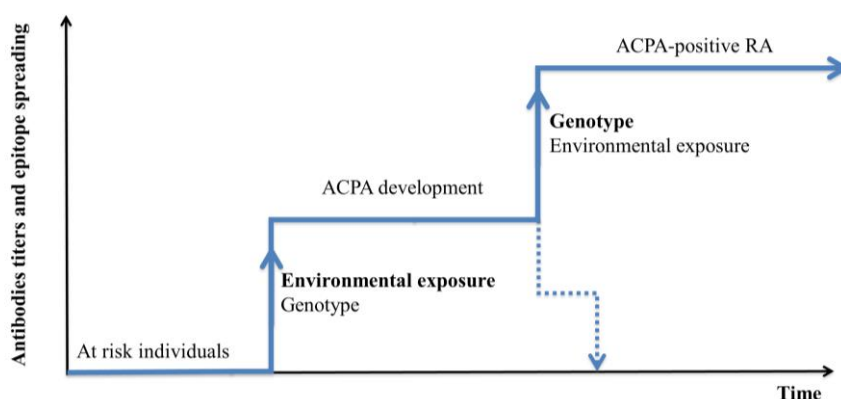
environmental factors was 88% (95% CI 57- 100) for ACPA without RA, 77.5% (95% CI 55-100) for ACPA, 57% (95% CI 26-99) for ACPA-positive RA and 61% (95% CI 34-95) for RA. Overall, all estimates had large confidence intervals and the confidence intervals for the estimated role of genetic factors included zero.

Heritability estimates for RA in different studies overlap. In comparison; the estimate from Twingene is in the lower range (39%) but it is supported by a large population based cohort using family similarities to calculate heritability (40%) [177]. Studies reporting higher heritability (>50%) likely represent the upper limit of heritability of RA as they conducted it in selected cohorts [180, 181], cohorts of treated/severe disease [181]. The specific heritability for ACPA-positive and/or RF-positive RA is less studied. However, the two studies reporting heritability in the range 45-50% were performed among more than >5000 RA cases using either family similarities or GWAS kinship analysis. Our data show heritability for ACPA-positive RA of 41% among fewer RA cases, but in a population-derived cohort, analyzed with a third method our data do support about 50% heritability for ACPA-positive RA.

We also found that non-shared environmental and unique environmental factors substantially contribute to the susceptibility for ACPA-positivity. This can be related to the findings in FDR cohorts showing normal or slightly raised prevalence of ACPA-positivity (excluding multicase-family cohorts) [189, 192, 196-199]. With regards to the prevalence of ACPA among twins, a Danish twin study in RA disease-discordant twin pairs reported high prevalence of ACPA among the healthy co-twins. The twin pairs in this study were in mean 60 years of age and about half of the RA-probands had developed RA before the age of 40. There was a significant increased risk for ACPA-positivity for healthy MZ co-twins compared to DZ co-twins, which suggested a large role of genetic factors for ACPA. One possible reason for the different finding in the Twingene cohort, with overall low prevalence of ACPA in RA disease-discordant twin pairs and the finding in the Danish cohort is the younger age of onset for RA in the Danish (mean age about 40 years) as compared to the Twingene cohort (median age at first registered RA diagnosis at 60 years). A recent study has suggested the genetical/familial influence on the risk for RA in relatives is increased if your relative develop RA before the age of 40 [177]. A vaccine study in twins have suggested that during optimized conditions the heritability of specific IgG response (anti-hepatitis A virus and anti-hepatitis B), is in the range of 36-61% (95% CI 0-81) suggesting an important role for none-shared environmental factors for inducing IgG-response [259]. Our result and the estimated heritability for ACPA-positivity of 23% (95% CI: 0-45) fit with this observed

variable role of genetic factors for inducing IgG response. Our result, showing that heavy smoking is a risk factor for ACPA-positivity in individuals without RA suggests that heavy smoking can be one of the initial environmental triggers. Based on our finding of stronger association between HLA-SE and ACPA-positive RA (with a characteristically strong ACPA response with high titers and many reactivities) compared with HLA-SE and ACPA-positive without RA (with a characteristically weaker ACPA response with low titers and few reactivities), and on the results from the heritability estimate showing weak influence of genetic factors for ACPA-development, we propose a new model for development of ACPA and RA. According to this, HLA-SE may be more important in determining which ACPA-positive individuals that eventually will develop RA (with a strong ACPA response) than determining which individuals will become ACPA-positive at onset before any symptoms of arthralgia/ arthritis (figure 8).

Figure 8: Model of stepwise development of ACPA



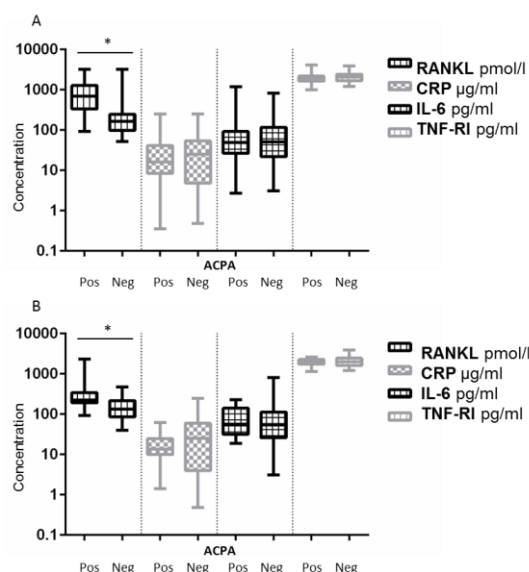
4.6 SERUM RANKL LEVELS ASSOCIATE WITH ACPA IN EARLY-UNTREATED RA

In study III we analyzed a cohort of early-untreated RA patients (N=183, 68% Anti-CCP positive; median age 52 years; 73% females) initiating MTX treatment and investigated serum levels of RANKL (Receptor activator of nuclear factor kappa B ligand) at baseline and after 3 months of treatment. We were interested in the relationship between RANKL and ACPA in RA, because RANKL is one of the key molecules in bone metabolism and is associated with bone turn-over [12, 13]. Studies of ACPA have suggested that bones might be directly affected by ACPA [93, 98, 106]. In parallel, studies of samples from RA patients antedating the diagnosis have shown an increase of some bone metabolism markers (OPG) but not others (such as RANKL (after adjusting for Anti-CCP) [260] and an association

between presence of ACPA and bone changes at RA diagnosis/onset [104, 128]. RANKL have been investigated in RA [11, 18, 19] and as a marker of bone damage [15, 20-22] but the influence of ACPA on RANKL in early RA was largely unexplored [261].

We found an increased level of RANKL levels in early RA that is associated with ACPA. ACPA-positive untreated early RA and ACPA-positive RF-negative untreated early RA had higher levels compared to ACPA-negative untreated early RA and ACPA-negative RF-negative untreated early RA. There were no differences in levels of the inflammatory markers CRP, IL-6 and TNF-RI (figure 9) associated with ACPA status.

Figure 9: Concentration of RANKL, CRP, IL-6 and TNF-RI in early-untreated RA
A) in ACPA-positive and ACPA negative patients; B) in RF-negative ACPA-positive and RF-negative ACPA negative patients; * $p < 0.05$.



In a small cohort, in which joint biopsies were available the synovial expression of RANKL was also increased in ACPA-positive compared to ACPA-negative early-untreated RA. Besides ACPA, age, BMI, and DAS28 associated with RANKL levels in peripheral blood but none of the other analyzed parameters (sex, smoking habits, ESR, CRP, IL-6 serum levels, TNF-RI serum levels, HAQ values, use of prednisolone or antiosteoporotic treatment, presence of HLA-SE and PTPN22 risk allele) did so. ACPA remained significantly associated with increased RANKL levels in the multivariate model including all significant predictors from the univariate analysis (unpublished data). The mechanisms explaining the association between increased levels of RANKL and ACPA are not investigated further in this study. ACPA might for example locally induce cytokine secretion such as TNF-alpha that affects RANKL secreting cells, such as osteocytes (reviewed in [262]). It is also possible that ACPA via direct mechanisms affect osteoclasts development without affecting RANKL and that the observed increased RANKL is a reflection of an overall dysregulated bone homeostasis characteristically for ACPA-positive RA.

4.7 SERUM RANKL LEVELS AND ACPA ASSOCIATE WITH BONE EROSIONS IN EARLY-UNTREATED RA

We find slightly increased RANKL levels in patients with evidence of bone erosions at baseline (median 413 pmol/l, IQR 263–1188, N=39) compared to those without bone erosions (382 pmol/l, IQR 195–871, N=144, p 0.11). The preliminary results from a recent study of RANKL in early RA (N=414), suggest a significant association between soluble RANKL levels and bone changes measured by a more sensitive method (Larsen score) [263]. When analyzing the RF negative patients in our cohort, and eliminating the bias of RF interference, RANKL serum concentrations were significantly increased in patients with evidence of bone erosions at baseline (median 243 pmol/l, IQR 194–284, N=9) compared with those without bone erosions (151 pmol/l, IQR 91–216, N=50). The relative difference in RANKL levels between erosive and non-erosive disease was in median a 40 % higher level in erosive as compared to non-erosive disease. Smoking habits were similar in patients with erosive disease and non-erosive disease. The biological relevance of this difference in peripheral blood and how it is mirroring the local level in bone or adjacent tissues, or levels of other influencing factors such as OPG the decoy-receptor for RANKL was not further investigated in this study. The synovial cohort was too small to investigate local synovial expression of RANKL in patients with erosions (N=2/15). However, differences in peripheral blood of similar magnitude have previously been shown to be of biological relevance for RA-associated bone destruction [11].

ACPA, measured as Anti-CCP2, is known to associate with both baseline and progressing bone changes in early RA [103, 264, 265]. To further investigate this association and the role of individual ACPA reactivities, we analyzed antibody reactivities to cit-enolase, -vimentin and -fibrinogen (and Anti-CCP2). We found significantly more prevalent baseline bone erosions in anti-cit-vimentin positive as compared to anti-cit-vimentin negative patients (32 vs. 15%, p <0.05). The association between anti-cit-vimentin antibodies and bone changes was confirmed in our validation cohort (N = 1,116) in which we found, that both Anti-CCP2-positive and anti-cit-vimentin positive patients had a higher frequency of bone changes in comparison to Anti-CCP-negative (31 vs. 22 %, p value <0.05) and anti-cit-vimentin negative patients (32 vs. 24 %, p value <0.05).

In contrast to this, previous studies have suggested, that Anti-CCP reactivity have the strongest association with bone changes. Among Anti-CCP positive patients it is reported, that specific ACPA reactivity doesn't modify the progression of bone changes [137, 154]. On the other hand, other studies suggest that anti-cit-vimentin (commonly co-occurring with

Anti-CCP) is particularly associated with bone changes [266-268]. A recent report show that early seroconversion from anti-cit-vimentin positive to negative is associated with less bone changes during two years follow up [137]. Further; additional observations during different phases of RA development suggest, that anti-cit-vimentin could be particular important or linked with the development of ACPA-positive RA (at least the bone destructive phenotype) as anti-cit-vimentin reactivity: a) typical is one of the first anti-cit reactivities to emerge in samples antedating RA diagnosis [58] b) increased occurrence is reported in FDR relative to RA [192, 197] c) is able to induce osteoclastogenesis [98] d) associates with severe extra-articular engagement in a cohort of long-standing RA [269]. This need to be further investigated as the typical ACPA response in RA patients is multiple and dissection of the role of individual reactivity is complicated. In addition, seroconversion needs to be taken into consideration when characterizing treated patients as antibody ‘seropositive’ or seronegative (particular for anti-cit-vimentin reactivity) (see below).

4.8 THE ACPA RESPONSE IN RA IS STABLE AND AFFECTED BY TREATMENT

Significant reductions in both RANKL and ACPA levels in sera were observed after 3 months of MTX treatment in a majority of the patients. The decrease in RANKL is in accordance with previous reports showing, that MTX decrease both synovial expression of RANKL in vivo and cellular expression of RANKL in vivo [17, 270, 271]. Similarly, the decrease in ACPA level is in accordance with previous reports in early RA. The relative changes (30-40%) we find in IgG ACPA seem similar with the change following anti-CD20 B-cell specific targeting therapy Rituximab [272, 273]. In parallel to the observed decreased serum concentrations of ACPA there were patients converting from seropositive to seronegative after 3 months MTX treatment. Anti-cit-vimentin and anti-cit fibrinogen reactivity was lost in about 30% of the patients. Anti-CCP2 and anti-cit enolase reactivity was more stable and lost in 3% respectively 6% of the patients. Conversion from negative at baseline to positive at 3 months was uncommon (anti-cit vim 3% and anti-cit fib 2 %). No conversion from seronegative at baseline to seropositive at 3 months was observed for ACPA or anti-cit enolase. The relative ‘instability’ of anti-cit-vimentin has been shown both during the development of RA (in Pre-RA samples possibly due to natural fluctuations [58]) and after treatment in newly diagnosed RA [137]. The reasons why and if anti-cit vimentin specific B-cells are particularly susceptible for treatment need to be investigated further. Additional investigations of how the ACPA response was affected by treatment with regards to changes in antibody affinity and how this further would modify suggested effector

mechanism by ACPA are also needed. Experimental studies showing specific functionality with isolated ACPA from treated established RA patients suggesting that treatment at least do not eliminate functionality [93].

4.9 FC-GLYCOSYLATION OF ANTIBODIES IN EARLY RA

In study IV we investigated Fc-glycans on IgG in early-untreated RA as a factor affected by and influencing the response to MTX treatment. Fc-glycans are important for the functionality of IgG, such as the binding to FcR and complement activation [113-115]. In this context, ACPA is suggested to have a changed pattern of Fc-glycosylation [63, 109, 117, 119].

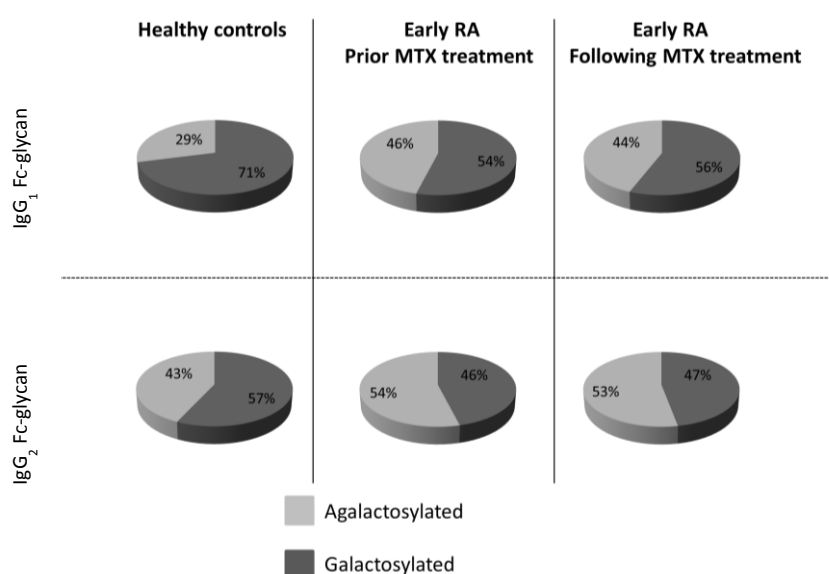
We analyzed sera from a cohort of 59 early-untreated RA patients (75% Anti-CCP positive and/or RF-positive, median age 53 years; 72% females) using a proteomic approach with specificity for IgG isotype and glycan substitution. We found a skewed IgG₁ and IgG₂ Fc-glycan pattern in RA patients, with 12 out of 19 characterized Fc-glycans being significantly different prior to MTX treatment compared to healthy controls (N =15). Among the Fc-glycans, the agalactosylated (lacking galactose) forms were more abundant ($p<0.05$) while the galactosylated (containing galactose) forms of IgG₁ and IgG₂ were less abundant ($p<0.05$) (Figure 10). Further analysis of other Fc-glycans (lacking fucose, with glycan bisection and/or with sialic acid) revealed that the difference between RA patients and controls were most pronounced for agalactosylated and galactosylated Fc-glycan. This finding can be added to a previous finding of a skewed galactosylation pattern of Fc-glycans in two untreated early RA cohorts [110, 274]. There seem to be a gradual change in Fc-glycosylation during development of RA, with increase in agalatosylated forms and decrease in galactosylated forms closer to, onset of RA [117, 119]. In treated RA the skewed agalactosylation pattern is characteristic and the ratio between agalactosylated and galactosylated glycans is correlated with disease severity [107, 108, 118, 274]

4.10 SUCCESSFUL MTX TREATMENT CHANGE THE IGG-FC-GLYCAN PATTERN IN EARLY RA

Treatment with MTX in our cohort partially changed the agalactosylation pattern. In patients responding (both good and moderate responders) to MTX at 3 months follow up (N=39) there was a decrease in agalactosylated (aGal) forms and increase in galactosylated(Gal) forms of IgG₁ and IgG₂. No significant change of aGal compared to Gal forms (aGal/Gal ratio) was noted in those not responding to MTX (N=20). Similar correlation between change in aGal/Gal ratio and improvement after 3 month of DMARD treatment has been reported in

a cohort of treated patients [275]. The specific proteomic method we used allowed us to further analyze the aGal/Gal ratio (including 12 different Fc-glycans) and we found that the decrease in the ratio of the most abundant agalactosylated form versus the most abundant mono- and digalactosylated forms (i.e the “main” aGal/Gal ratio) of IgG1 was the most pronounced compared to aGal/Gal forms lacking fucose (including 3 other of the 9) or with glycan bisection (including the last 3 of the 9). Overall, the ‘main’ aGal/Gal forms constituted about 80% of the IgG1 Fc-glycan profile.

Figure 10: Agalactosylated and galactosylated IgG₁ and IgG₂ Fc-glycans in healthy controls and early RA prior and following treatment.



Abundance of agalactosylated and galactosylated Fc-glycans were significantly differed ($p < 0.05$) in healthy controls compared to early RA.

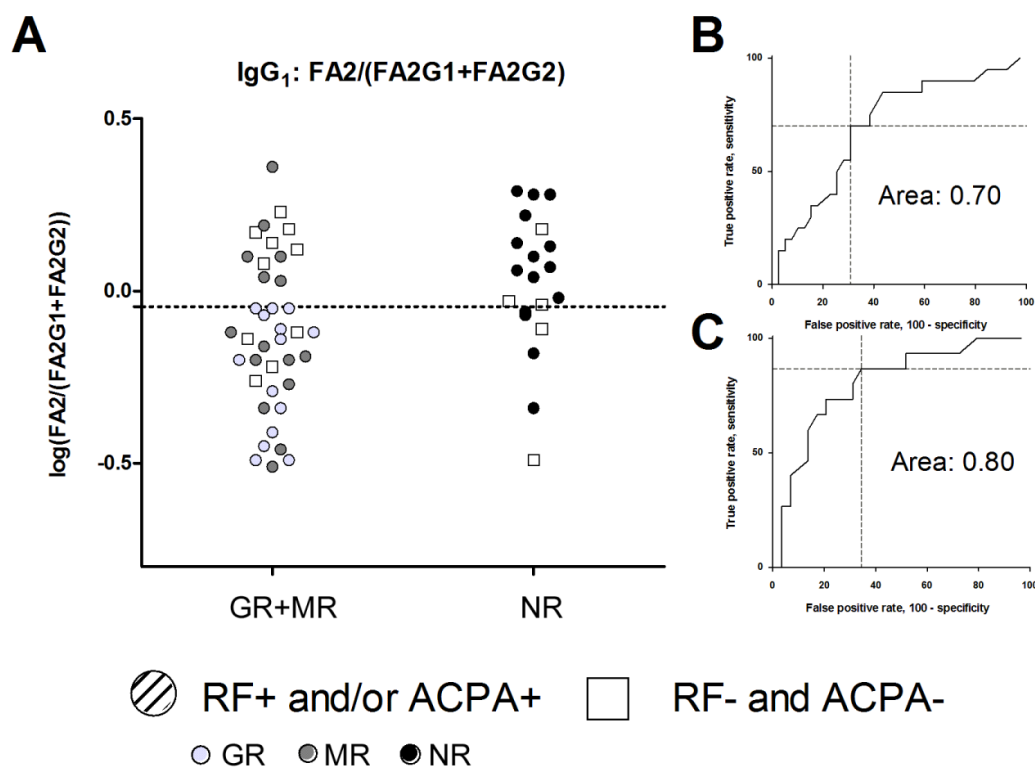
4.11 IGG-FC-GLYCAN PATTERN DISTINGUISH RESPONDERS AND NON-RESPONDERS

In order to study in more detail the Fc-glycans of IgG and other factors that associated with MTX-responding patients and MTX-non-responding patients we performed multivariate statistical modeling to find a model that could distinguish responders from non-responders. We included information about baseline characteristics; sex, age, smoking; baseline and intra-individual changes of antibody Fc-glycans, IgG-Fc protein isotype, DAS, HAQ and CRP levels. In the model including all patients, misclassifications were common among seronegative (ACPA-negative and RF-negative) patients. The predictive ability increased in a model including only seropositive patients. When separately looking into the different factors, changes in the main aGal/Gal ratio of IgG₁ and DAS-score were prominent factors

associated with lack of response in both models. In the model with seropositive RA patients the baseline aGal/Gal ratio of IgG1 was an additional prominent factor associated with lack of response.

As baseline IgG₁ ‘main’ aGal/Gal ratio and change of this ratio had the highest ranking of the glycan factors distinguishing between responders and non-responders, we further estimated its performance in predicting therapy response. We defined a cut-off using a ROC curve and found that the IgG₁ ‘main’ aGal/Gal ratio could discriminate future non-responders from responders to both MTX therapy with a sensitivity of 70% (95% CI: 46-88%) and a specificity of 69% (95% CI: 52-83%). In seropositive patients, sensitivity was increased to 80% (95% CI: 52-96%) with a specificity slightly lowered of 69% (95% CI 49-85%) (Figure 11).

Figure 11: The galactosylated ‘main’ aGal/Gal ratio of IgG₁ as a predictive factor for response.



A) The ‘main’ aGal/Gal ratio of IgG₁ in good and moderate responding (GR+MR) and none-responding patients (NR). ROC curve based on distinguishing (B) GR+MR versus NR (C) among ACPA-positive and/or RF-positive patients.

Based on this, we concluded that ‘main’ aGal/Gal ratio of IgG₁ could have predictive value for MTX response in early RA, but this needs to be validated in a larger cohort. The additive value compared to CRP will also need to be investigated further, as we found that baseline CRP was a predictor for response but weaker in our cohort. Disease activity as

measured by DAS28 didn't have predictive value suggesting that 'main' aGal/Gal ratio may reflect disease processes partly uncoupled from inflammation. In a future validation cohort information about bone destructions and complement activation could be co-analyzed as experimental studies suggest both these effects of Fc-galactosylation is suggested in experimental studies [115, 116, 276]. One previous study failed to find any predictive value of aGal/Gal in treated RA [275]. Potential reasons for this discrepancy are differences in study design (such as analysis of early-untreated patients versus treated patients, the use of MTX contra anti-TNF, and the method allowing detailed versus more crude characterization of the glycan patterns).

5 CONCLUSIONS

- A) Low concentration and few ACPA reactivities are common in ACPA-positive individuals without RA as compared with patients with RA. Presence of ACPA, in particular high titers of Anti-CCP2 have a high diagnostic accuracy for an RA diagnosis in a population-derived cohort. Heavy smoking and HLA-SE are possible risk factors associated with development of ACPA. Environmental factors are important for the variability in ACPA status and heritability of ACPA-positive RA is about 50%.
- B) The ACPA response in RA is stable but seroconversion is relatively common for reactivity to cit-vimentin. ACPA and specifically anti-cit-vimentin antibodies associate with bone destruction in early-untreated RA.
- C) Serum RANKL associate with ACPA and bone destruction in early-untreated RA and is affected by treatment.
- D) Fc-agalactosylation of IgG antibodies is common in early RA compared to controls and is modified after successful MTX treatment. A baseline ratio between Fc-agalactosylation and Fc-galactosylation of IgG₁ can distinguish between responders and non-responders and is a candidate predicting factor of MTX response for future studies.

5.1 FUTURE DIRECTIONS

There are several potential future continuations of the current studies and I will just highlight a few that I find of particular interesting. In general there is a need to further understand the involvement of environmental factors in the development of ACPA and ACPA-positive RA as these can be separate. A better understanding of the risk and protective factors involved in the transition from being ACPA-positive without specific joint symptoms to being ACPA positive with arthralgia is especially important, as this transition seem to be a window of opportunity for preventing RA. Establishment of prospective screening-cohorts (for research purpose) with ACPA-positive individuals and follow-up samples would allow an investigation of the nature of the ACPA response in more detail. Influence by ACPA both during the antedating period and after onset on the actual RA phenotype could be investigated. Both experimental and observational studies are needed. What about ACPA-negative RA? I believe and hope that increasing and detailed understandings of ACPA-positive RA will lead to better understandings of ACPA-negative RA as well.

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Herta & Vigdis; Ni är det finaste som finns, ni är det dyraste i världen, ni är som stjärnorna, som vindarna, som vågorna, som fåglarna, som blommorna på marken

L; Du har sat dig på tvärs i mit univers! I am so happy to share my life with you. Thank you for love, discussions and everything.

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